CHARACTERIZATION OF NOVEL NEURAL STEM CELL POPULATIONS IN THE *DROSOPHILA* CENTRAL NERVOUS SYSTEM

by

JASON NATHANIEL BOONE

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Confirmation of Approval and Acceptance of Dissertation prepared by:

Jason Boone

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This dissertation has been accepted and approved in partial fulfillment of the requirements for the Doctor of Philosophy degree in the Department of Biology by:

Eric Johnson, Chairperson, Biology
Christopher Doe, Advisor, Biology
Judith Eisen, Advisor, Biology
Charles Kimmel, Member, Biology
Kenneth Prehoda, Outside Member, Chemistry

and Richard Linton, Vice President for Research and Graduate Studies/Dean of the Graduate School for the University of Oregon.

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Approved: ______________________________________
Dr. Chris Doe, Advisor

Neuroblasts are the neural stem cells of the *Drosophlia* central nervous system. They are large cells that divide asymmetrically to renew another neuroblast and generate a smaller ganglion mother cell (gmc) that will divide once to produce two neurons. Combining genetic lineage tracing experiments with cell fate markers I isolated two separate neural stem cell populations with distinct locations and cellular behaviors in the larval brain. In my first chapter I introduce the central nervous system of *Drosophila* and in the next two sections of chapter I, I introduce the development of the optic lobe and central brain, two separate structures of the central nervous system. In my second chapter I characterize the lineage relationship of cells within the developing larval optic lobe and use cell fate markers to determine the identity of these cells. Next I examine the effect of spindle orientation on cell fate within epithelial cells of the optic lobe. In my third chapter I characterize another novel neural stem cell lineage in the larval brain containing GMCs

with greater proliferation potential than a "canonical" GMC, and I term these, transit amplifying gmcs (TA-GMCs). Further I show that the parent neuroblast of these novel TA-GMCs does not asymmetrically segregate the fate determinant Prospero (Pros) thereby producing a GMC with greater proliferation potential. Finally I show that TA-GMCs do asymmetrically segregate the fate determinant Pros, divide slowly and give rise to up to 10 neurons which normal gmcs never do. In my fourth chapter I show preliminary work on the characterization of a mutation that causes excessive production of neuroblasts specifically in novel TA-GMC lineages. These findings reveal novel neural stem cell lineages, patterns of asymmetric cell division and patterns of neurogenesis that could aid in our understanding of neural stem cell biology and tumorogenesis. This dissertation includes both my previously published and my co-authored materials.

CURRICULUM VITAE

NAME OF AUTHOR: Jason Nathaniel Boone

PLACE OF BIRTH: Sodus, New York

DATE OF BIRTH: September 15, 1976

GRADUATE AND UNDERGRADUATE SCHOOLS ATTENDED:

University of Oregon, Eugene, Oregon
University of Vermont, Burlington, Vermont
Alfred University, Alfred, New York

DEGREES AWARDED:

Doctor of Philosophy in Biology, 2008, University of Oregon
Bachelor of Arts, 1999, Alfred University

AREAS OF SPECIAL INTEREST:

Stem Cell Biology
Cell Biology
Developmental Biology

PROFESSIONAL EXPERIENCE:

Graduate Research Fellow, Department of Biology, University of Oregon, Eugene, Oregon, 2003-2008

Graduate Teaching Fellow, Department of Biology, University of Oregon, Eugene, Oregon, 2002-2003

Research Technician, Dr. Diane Jaworski Lab, University of Vermont, Burlington Vermont, 2001-2002

Research Technician, Dr. Sally Huber Lab, University of Vermont, Colchester Vermont, 2000-2001

GRANTS, AWARDS AND HONORS:

Howard Hughes Predoctoral Funding, University of Oregon, 2007-2008

NSF Integrative Graduate Education and Research Traineeship (IGERT) Training Grant, University of Oregon, 2003-2004, 2005-2007

PUBLICATIONS:

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I dedicate this document to my parents, who have always supported my endeavors, scientific and otherwise.

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CHAPTER I

INTRODUCTION TO THE CENTRAL NERVOUS SYSTEM AND ITS DEVELOPMENT

Background on the Central Nervous System

One of the fundamental questions in biology today is how metazoan animals develop a complex central nervous system. The central nervous system (CNS) of metazoan animals is an extraordinary organ capable of: 1) perceiving external stimuli, 2) processing the given stimuli and 3) executing an appropriate response based upon the given stimulus. Since the mammalian CNS is a highly complex and integrated system with many thousands of different cell types, a careful examination of an organism with less cells and fewer cell types would be advantageous. For this reason I utilized the arthropod, *Drosophila melanogaster*, as a model system to understand how the multitude and number of cells and cell types are formed during CNS development.

The adult *Drosophila melanogaster* CNS, in general terms, is composed of two brain lobes, two optic lobes and a ventral nerve cord (VNC). These brain regions are homologous to the mammalian brain with two cortical hemispheres and a spinal cord. In *D. melanogaster* the two brain lobes and optic lobes are located within the head exo-skeleton, similar to the brain of mammals, whereas the VNC is located within the thoracic body region (Meinertzhagen et al., 1998).

The brain lobes of *Drosophila* have many functions, some of which are well characterized: memory, flight control and olfaction (Vosshall et al., 2007). These behaviors are the outputs of specific structures within the brain lobes of the fly. For instance, the mushroom body has been implicated in learning and memory (Liu et al., 2006), whereas the central complex, which includes the protocerebral bridge, fan-shaped body, ellipsoid bodies and ventral bodies has been shown to control fine motor walking movements and flight trajectory (Ilius et al., 2007). The antennal lobe, located in the anterior compartment of the brain lobe nearest the antennae, is the primary olfactory association center where smells are processed (Laissue et al., 1999).

Likewise, the optic lobe is a highly complex structure located at each lateral surface of the adult brain. It is situated between the central brain and directly beneath the overlying retina, precisely positioned to modulate visual processing. Neurons from the outer retina enter the optic lobe and cross over within the optic chiasm. From here neurons project through the lamina into the medulla. The optic lobe is a highly stratified and modular structure exquisitely wired for the rapid visual processing in flying insects (Ting et al., 2007). Because the finely tuned neuronal structures of the optic lobe yield such behaviors, it is natural to wonder how such a system develops. I provide a short overview of the development of the central nervous system in several organisms below. Following that I introduce the development of the optic lobe and larval central brain neuroblast lineages.

Background on Central Nervous System Development

The Drosophila central brain develops from large (>8um) neural precursors called neuroblasts, that delaminate from overlying head neuroepithelium in early embryonic stages (Doe, 2008). Each brain lobe contains roughly 100 central brain neuroblasts that proliferate throughout embryonic stages generating the larval central brain. Toward the end of embryogenesis all but five central brain neuroblasts enter a period of quiescence; four mushroom body (MB) neuroblasts and one antennal lobe (AL) neuroblast do not enter quiescence at late embryonic stages, that continue to proliferate through the quiescent period. During larval stages the remaining ~95 neuroblasts slowly exit quiescence and begin to proliferate and together with the MB and AL neuroblasts, generate the nervous system of the adult central brain (Ebens et al., 1993). At each larval stage there are a defined number of central brain neuroblasts that have begun to proliferate and to express neuroblast specific markers. Neuroblasts continue to proliferate through larval stages into early pupal stages when many stop dividing. However, there is a small population of neuroblasts that remains proliferative into late pupal stages (J.Q.B unpublished observations). Whether or not these late proliferating neuroblasts are the mushroom body neuroblasts in unclear. Furthermore, it is unclear whether these neuroblasts terminally differentiate or die when they reach the ends of their lineages.

D. melanogaster mushroom body (MB) neuroblasts are the progenitors of the adult mushroom body. The mushroom body is one of the few systems for understanding how neuronal progenitors give rise to adult structures and it has been well characterized (reviewed in Doe, 2006). Four MB neuroblasts divide asymmetrically to renew another

MB neuroblast and to give rise to a ganglion mother cell (gmc). Each ganglion mother cell then divides once to generate two neurons (Doe, 2008). Neurons generated by the MB neuroblasts are born in a temporally specific pattern such that early born neurons project to the gamma lobe, later born neurons project to the alpha'/beta' lobe and late born neurons project to the alpha/beta lobe (Liu et al., 2006). This mode of developing specific neuronal structures is similar to the development of the *D. melanogaster* and *Schistocerca* embryonic nerve cord (Kuwada et al., 1985). In this way, the highly ordered structure of the adult MB and CNS are constructed.

In the honey bee, *Apis mellifera carnica*, the mushroom body consists of many more neurons or Kenyon cells (~2500 in *D melanogaster* vs 170,000 in *A mellifera*) than are found in *D melanogaster* (Malun, 1998). It has been proposed that, if the four individual MB neuroblasts of the honey bee were to generate all of the 170,000 neurons through individual asymmetric divisions, the parent MB neuroblast would need to undergo 137 divisions per hour; roughly equivalent to 42,500 asymmetric divisions (Malun, 1998). Instead, Damar Malun has suggested that Kenyon cells are generated by symmetric dividing progeny of parent MB neuroblasts. Indeed, he has shown large clusters of cells incorporate BrdU nearest the parent MB neuroblast indicating the progeny of MB neuroblasts are proliferative. In this way, the progeny of the mushroom body neuroblasts undergo multiple rounds of division in order to generate the large number of Kenyon cells needed for the honey bee mushroom body.

Interestingly, of amplification of stem cell progeny is often employed by other organisms with large, complex nervous systems. For example, mammals utilize "transit-

amplifying" (TA) progenitors to generate many neurons from a single, slowly dividing neural stem cell. TA progenitors have been found in other organ systems, including the developing and regenerating skin of mammals (Waters et al., 2007).

Although the exact mechanisms remain still unclear, another way of generating large numbers of neuronal progeny can be found in the vertebrate neural tube. During the development of the vertebrate neural tube, neuroepithelial progenitors fold into a tube along the anterior-posterior axis of the organism. It is hypothesized that neuroepithelial progenitors then undergo multiple rounds of division to increase to pool of progenitor cells. Slowly, radial glial cells are born from surrounding neuroepithelial cells whereupon radial glia generate the neurons of the spinal cord (Gotz et al., 2005). Currently it is not know whether neuroepithelial cells can give rise to neurons directly or whether there must always be a radial glial progenitor. In any event, the vertebrate neural tube utilizes a slightly different strategy to generate the thousands of neurons needed; early progenitor cells divide rapidly to generate a large pool size, then later transform into another cell type, a "neuronal progenitor" to generate the thousands of neurons necessary (Gotz et al., 2005).

Background on Drosophila Optic Lobe Development

In contrast to embryonic and MB neuroblasts, a third class of neuroblasts, those residing in the optic lobe, has been less well characterized. The optic lobe derives from an embryonic optic placode that invaginates into the embryo (Green et al., 1993). The optic lobe cells start to proliferate soon after larval hatching and separate into an outer

proliferation center (OPC) and an inner proliferation center (IPC). The OPC generates the outer medulla and lamina neurons; the IPC generates the inner medulla, lobula and lobula plate neurons (Meinertzhagen et al., 1993). It has been reported that the early optic lobe cells are neuroblasts that divide symmetrically to expand the population and that these cells later switch to asymmetric divisions to produce the neurons of the visual system (Ebens et al., 1993; Ceron et al., 2001). An alternative hypothesis suggests that early optic lobe cells comprise a symmetrically dividing epithelial sheet that later generates asymmetrically dividing neuroblasts by an unknown mechanism (White et al., 1978; Hofbauer et al., 1990; Nassif et al., 2003). However, the lineage relationship between cell types of the optic lobe has never been directly determined. Thus it is formally possible that the early symmetrically dividing epithelial cells and later developing asymmetrically dividing neuroblasts are two separate cell pools that do not contribute to each other.

Within the optic lobe, the development of the lamina has been well studied. Laminar neurons are born from the most lateral edge of the optic lobe epithelium. Epithelial progenitors undergo two rounds of cell division before directly differentiating into laminar neurons (ref). The origins of the highly stratified medulla however, have not been studied in any detail. Open questions regarding the development of the medulla include: 1) where do medulla neurons come from, 2) what is their mode of birth, 3) do optic lobe progenitors start as neuroblasts or epithelial cells and 4) do optic lobe epithelial cells give rise to optic lobe neuroblasts?

Background on Drosophila Central Brain Neuroblasts

Larval central brain neuroblasts have recently become a model for studying neural stem cell self-renewal (Bello et al., 2006; Betschinger et al., 2006; Lee et al., 2006a,b,c; Wang et al., 2006; Doe, 2008). Neuroblasts divide asymmetrically in cell size and fate to renew a larger neuroblast and a smaller ganglion mother cell (GMC). The neuroblast continues to proliferate, whereas the GMC typically generates just two post-mitotic neurons (Goodman and Doe, 1993; Lee and Luo, 1999; Pearson and Doe, 2003). Many proteins are asymmetrically segregated during neuroblast mitosis: the apical proteins Bazooka, aPKC, Par-6, Partner of Inscuteable (Pins), and Inscuteable (Insc) are segregated into the neuroblast, whereas the basal proteins Numb, Miranda (Mira), Prospero (Pros), and Brain tumor (Brat) are localized into the GMC (reviewed in Caussinus and Hirth, 2007). aPKC promotes neuroblast self-renewal, whereas the basal proteins Numb, Mira, Brat, and Pros all act to inhibit self-renewal and promote neuronal differentiation (Bello et al., 2006; Betschinger et al., 2006; Choksi et al., 2006; Lee et al., 2006a,c; Wang et al., 2006).

Neuroblast transcription factors include the basic-helix-loop-helix protein Deadpan (Dpn), which promotes optic lobe proliferation (Wallace et al., 2000), but has not been assayed for a role in neuroblast proliferation. In contrast, the Pros transcriptional repressor is nuclear in GMCs and young neurons (Hirata et al., 1995; Knoblich et al., 1995; Spana and Doe, 1995; Li and Vaessin, 2000), where it down regulates cell cycle gene expression to restrict GMCs to one terminal mitosis (Hirata et al., 1995; Knoblich et al., 1995; Spana and Doe, 1995; Li and Vaessin, 2000).

In many mammalian tissues, stem cells generate lineage-restricted ''transit amplifying cells'' that can proliferate to expand the number of differentiated progeny made from a single precursor (Morrison and Kimble, 2006; Nakagawa et al., 2007). Teasing out the mechanisms that regulate stem cell proliferation and self-renewal from those regulating proliferation of transit amplifying progenitors is an important goal of stem cell biology, and has been complicated by the difficulty in identifying each type of progenitor in vivo or in vitro. Open questions include: 1) Do transit amplifying progenitors exist in the developing *Drosophila* central brain, 2) What are the origins of transit amplifying progenitors in *Drosophila* 3) Do transit amplifying progenitors assist in producing more progeny over a shorter period of time.

Bridge to Chapter II

In the preceding chapter, I outlined central questions on the development of the central nervous system and optic lobe, including the current understanding of cell fates within the central brain and optic lobe, as well as the current understanding of division modes within the central brain and optic lobe. In Chapter II, I will use my previously published co-authored data to describe the cell fates within the optic lobe, including how the cells within the developing optic lobe are related. I will also show how spindle orientation does not affect cell fate within the developing optic lobe epithelium. I will show that the development of the optic lobe is similar to vertebrate neural tube development and how the optic lobe can be used as a model system to understand vertebrate neural epithelial stem cell development.

CHAPTER II

REGULATION OF SPINDLE ORIENTATION AND NEURAL STEM CELL FATE IN THE *DROSOPHILA* OPTIC LOBE

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* These authors contributed equally to this work.

BACKGROUND

The division modes of stem cells are tightly regulated during development and adult tissue homeostasis. This ensures that tissues and organ systems develop to the correct size and contain the correct cell types for proper function. One way to expand the pool of stem or progenitor cells during development is to undergo symmetric cell division. Conversely, one way to generate differentiating cell types, while maintaining a constant stem/progenitor population, is to undergo asymmetric cell division where one daughter differentiates and the other remains a stem cell [1]. Recently, it has been suggested that the ratio of stem/progenitor cells to differentiating cells in a tissue can be regulated by changing spindle orientation, thereby altering the proportion of symmetric to asymmetric cell divisions. For example, it has been proposed that mammalian neuroepithelial cells first expand via symmetric divisions, followed by a burst of neuron production resulting from asymmetric divisions [2]. Recently, it has been reported that altering the division axis in several different vertebrate cell types can lead to a change in

fate, for example, in mammalian basal epidermal cells, neural progenitor cells in the developing neocortex and progenitors in the developing retina [3-5]. Despite the recent advances in understanding stem cell self-renewal and spindle orientation in both mammalian and *Drosophila* systems [6], however, very little is known about the relationship between spindle orientation and cell type specification. Do stochastic changes in spindle orientation generate cell diversity during normal development, or does spindle orientation always respond to cell type specification?

In *Drosophila*, the central nervous system is derived from neural stem cells called neuroblasts. There are at least three types of neuroblasts: embryonic, larval central brain/thoracic, and larval optic lobe. They all undergo asymmetric cell division, self-renewing the neuroblast while producing a differentiating daughter cell (ganglion mother cell; GMC). Embryonic neuroblasts delaminate as single cells from a polarized epithelium called the ventral neuroectoderm. Whereas neuroectodermal cells divide symmetrically with a horizontal mitotic spindle (in the plane of the neuroectoderm), neuroblasts rotate their spindles to a vertical plane (perpendicular to the neuroectoderm) and divide asymmetrically to generate a large apical neuroblast and a smaller basal GMC. The GMC typically generates two post-mitotic neurons. Embryonic neuroblast divisions are molecularly and physically asymmetric: the neuroblast inherits apical proteins (for example, atypical Protein kinase C (aPKC) and Inscuteable (Insc)) and the GMC inherits basal proteins (for example, Miranda (Mira), Prospero (Pros), Numb, and Partner of Numb (Pon)) [7]. Larval central brain/thoracic neuroblasts derive from embryonic neuroblasts and undergo a similar asymmetric cell division along their apical/basal axis

of polarity. Progress has been made in understanding the molecules that are involved in the self-renewing capacity of larval central brain neuroblasts, and of how misregulation of these factors can lead to tumor formation [8-12]. However, little is known about symmetric divisions in the nervous system and what the molecular switch is that leads to asymmetric division.

In contrast to embryonic neuroblasts and larval central brain neuroblasts, the third class of neuroblasts, those residing in the optic lobe, has been less well characterized. The optic lobe derives from an embryonic optic placode that invaginates into the embryo [13]. The optic lobe cells start to proliferate soon after larval hatching and separate into an outer proliferation center (OPC) and an inner proliferation center (IPC). The OPC generates the outer medulla and the lamina neurons; the IPC generates the inner medulla, the lobula and the lobula plate neurons [14]. It has been reported that the early optic lobe cells are neuroblasts that divide symmetrically to expand the population and then later switch to asymmetric division to produce the neurons of the visual system [15,16]. An alternative hypothesis suggests that early optic lobe cells comprise a symmetrically dividing epithelial sheet that later generates asymmetrically dividing neuroblasts by an unknown mechanism [17-19]. However, the lineage relationship between cell types of the optic lobe has never been directly determined, and it is formally possible that the early symmetrically dividing epithelial cells and later developing asymmetrically dividing neuroblasts are two separate cell pools that do not contribute to each other.

Here we use newly available molecular markers, live imaging methods, and genetic lineage techniques to investigate the relationship between symmetrically dividing

early progenitors and the asymmetrically dividing neuroblasts of the optic lobe. We test whether changes in spindle orientation are sufficient to induce neuronal differentiation, as has been inferred for the mammalian retina [5]. We find that optic lobe neuroblasts derive from the lateral optic lobe neuroepithelium; that there is a transition from symmetric to asymmetric stem cell-like divisions between these two progenitor populations; and that inducing vertical spindle orientation in neuroepithelial cells is not sufficient to generate ectopic neuroblasts or neurons. Therefore, spindle orientation does not determine cell fate, but is itself regulated in response to cell type specification.

RESULTS

Optic lobe morphogenesis

We screened a collection of GAL4 enhancer trap lines to identify markers for optic lobe cell types. The expression of one line, $GAL4^{c855a}$ [20,21], is restricted to the optic lobes (Figure 1). We used this line to drive expression of *UAS-partner of numb-gfp* (*pon-gfp*) [22] and followed optic lobe morphogenesis throughout larval development (Figure 1). Frontal brain confocal sections show that, at mid third instar, the developing OPC of the optic lobe forms a dome-shaped shell covering the lateral brain lobe with an opening pore at its center, while the IPC is U-shaped with the opening of the U pointing in the dorso-caudal direction (Figure 1a, b) [18]. This structure arises from a small group of 30 to 40 progenitor cells in newly hatched larvae [18]; by 24 hours after larval hatching (ALH) the OPC and the IPC can be distinguished (Figure 1c) and each population forms an expanding epithelial sheet throughout larval development (Figure

1d–f). By the second instar larval stage, a population of cells at the medial edges of the OPC epithelium appears to round up, loses epithelial morphology, and down-regulates *GAL4*^{c855a}. These are likely to be the previously described OPC neuroblasts [18,19].

The optic lobe consists of two distinct cell types

Previous studies have drawn different conclusions about the cell types of the optic lobe. Some reports suggest that the early optic lobe consists initially of symmetrically dividing neuroblasts that, at later stages, become asymmetrically dividing neuroblasts [15,16]. In contrast, other reports conclude that the early optic lobe consists of epithelial cells and only later do neuroblasts develop at the medial edges of the epithelium [18,19]. In the latter studies it has been assumed that the optic lobe neuroblasts derive from the optic lobe epithelium, but this has never been tested directly by lineage studies. In this section and the following one, we discuss the use of molecular markers, live imaging experiments, and genetic cell lineage analysis to resolve the identity and origins of these optic lobe cell types. We first tested whether the optic lobe contains epithelial cells by staining for epithelial junctional marker proteins. PatJ is a cytoplasmic scaffolding protein and is part of the conserved Crumbs complex, which is located in apical and subapical regions in epithelial cells. DE-Cadherin (DE-cad) is a transmembrane protein located at the zonula adherens, while Discs large (Dlg) and Scribble (Scrib) are PDZ domain tumor suppressor proteins that are enriched at the basolateral septate junctions [23]. We found that a subpopulation of the optic lobe cells, those that express *GAL4*^{c855a}

and have epithelial morphology (Figure 1), express all of these junctional markers, and that they localize to their appropriate cellular domains (Figure 2a). Thus, the optic lobe contains an epithelial cell population that expands during early larval stages and becomes depleted by pupariation (Figure 1).

To determine if these epithelial cells have neuroepithelial features, we assayed for the expression of the proneural genes *achaete* (*ac*) and *scute* (*sc*). *ac* and *sc* are expressed in clusters of cells in other epithelia (for example, embryonic ventral ectoderm and imaginal discs) where they promote neurogenesis. Delta-Notch signaling antagonizes proneural expression, resulting in only one or a few cells in the cluster developing as a neuroblast (embryo) or a sense organ precursor (imaginal disc), while the remaining cells adopt an epidermal fate [24,25]. We found that all cells in the OPC express the proneural gene *scute* (Figure 2b; data not shown), but we observed no expression of the proneural gene *achaete* (data not shown). Thus, the optic lobe epithelium is a neuroepithelium and all cells in the epithelial sheet appear to have the potential to enter the neural pathway.

We next assayed neuroblast markers, to determine if the neuroepithelial cells are actually neuroblasts undergoing symmetric divisions to expand the neuroblast population [15,16]. We stained for Deadpan (Dpn) and Mira, which label all known embryonic and larval central brain neuroblasts [9-11,26,27] and found that these markers failed to label the neuroepithelial cells of the optic lobe (Figure 2b–e). They did, however, label a population of rounded cells at the edge of the epithelium, which lacked Dlg/Scrib septate

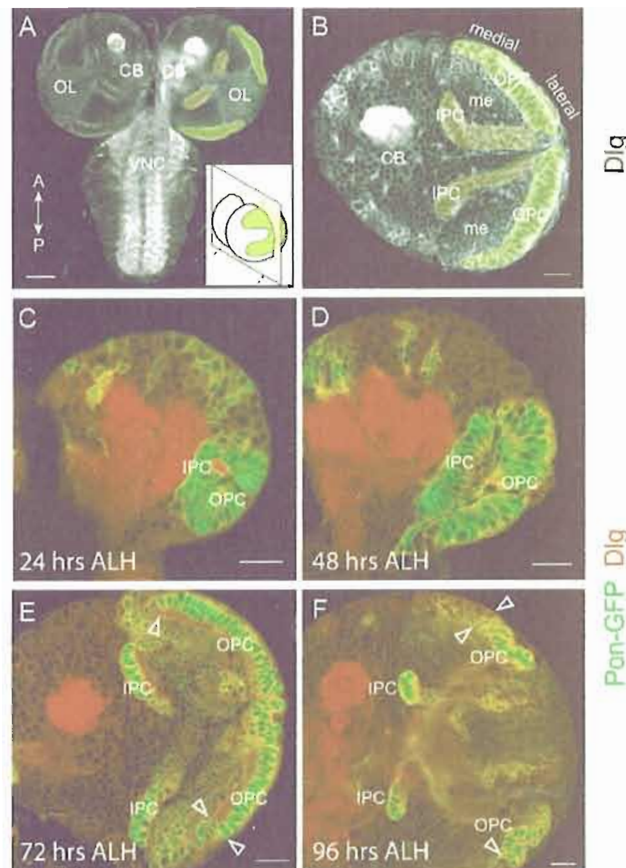

Figure 1. *GAL4*^{c855a} reveals the proliferation centers of the developing optic lobe. (**a**) A late third instar larval central nervous system (CNS): ventral nerve cord (VNC), central brain (CB) and optic lobes (OL). Subsequent images show frontal confocal sections, as shown in the inset diagram (OPC in green). Anterior and posterior refer to the neuraxis of the larval CNS. (**b**) A frontal section through a brain lobe at mid third instar: the OPC (green), the inner proliferation centre (IPC, yellow) and the medulla cortex (me). Discs large (Dlg; grey) outlines all cell cortices in the larval brain and highlights the morphology of the two optic lobe proliferation centers. (**c**) *GAL4*^{c855a} begins to drive expression of *UAS-pon-gfp* (green; Dlg in red) at first instar. At late first/early second instar (24 hours ALH; after hatching), the OPC and the IPC can be distinguished as two closely associated epithelia. The cells belonging to the proliferation centers (green) are clearly distinguishable by their columnar shape, in contrast to the round, isolated central brain cells. (**d**) At the end of second/early third instar (48 hours ALH) the epithelia of the OPC and IPC separate from each other and smaller progeny cells are located between the two epithelia. (**e**) As development progresses during second to mid third instar (72 hours ALH) the OPC cells at the medial edge of the epithelium loose their columnar shape (to the left of the arrowheads). (**f**) At late third instar (96 hours ALH) the OPC epithelium decreases in size while the number of round neuroblast-like cells increases at the medial edges (to the left of the arrowheads). All images are single confocal sections, with anterior on top and lateral to the right. Scale bar is 50 μm (a) and 20 μm (b-f).

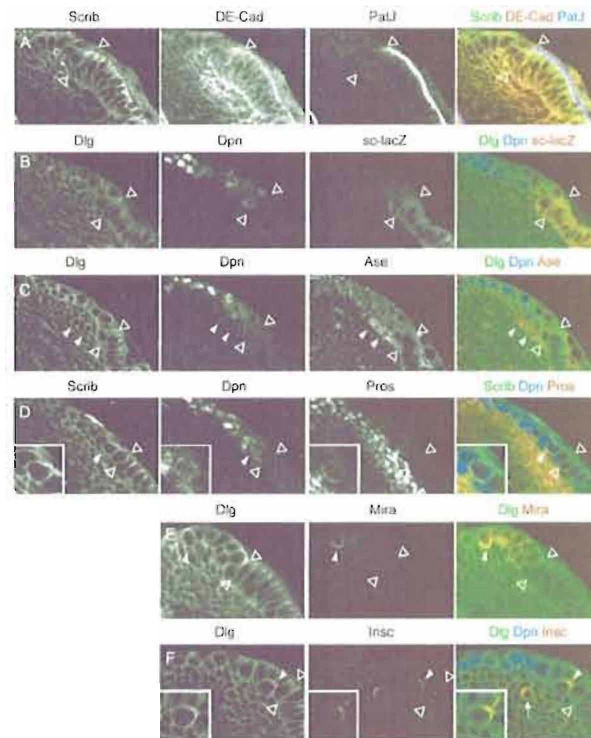

Figure 2. Optic lobe neuroepithelial cells and neuroblasts are arranged in distinct medio-lateral zones. (**a**) The developing optic lobe generates a lateral epithelial region (to the right of the arrowheads). Epithelial cells express three proteins that localize to cellular junctions: Scrib (green) basolateral septate junctions in epithelial cells; DE-Cad (red) basolateral zonula adherens; and PatJ (blue) apical and subapical regions in epithelial cells. Medial neuroblasts (to the left of the arrowheads) are round and lack subcellular localization of junctional proteins. (**b**) *sc-lacZ* (red) is expressed in the lateral epithelium of optic lobe (to the right of the arrowheads). Expression diminished in medial optic lobe neuroblasts (to the left of the arrowheads). Medial neuroblasts express the bHLH transcription factor Dpn (blue), which is not expressed in neuroepithelial cells. Dlg (green) outlines all cell cortices but enriched at adherens junctions. (**c**) Asense (red) shows weak cytoplasmic expression in medial Dpn (blue) positive neuroblasts (to the left of the open arrowheads). Asense is nuclear in the progeny of neuroblasts (filled arrowheads). (**d**) Pros protein (red) forms a basal crescent (inset) in mitotic medial optic lobe neuroblasts (filled arrowhead). Dpn (blue) is restricted to the neuroblasts but Pros (red) is inherited by the basal progeny cells. (**e**) Mira (red) forms a basal crescent in mitotic neuroblasts (filled arrowhead) (metaphase; n = 9 and telophase n = 9). (**f**) Insc protein (red) forms an apical crescent in mitotic medial optic lobe neuroblasts (filled arrowhead and inset). These neuroblasts reveal weak cytoplasmic Dpn (blue). Dlg (green) is enriched apically, where it co-localizes with the Insc crescent at the apical cortex (inset). Some progeny cells in the medulla cortex also express *insc* (arrow). All images are single confocal sections from third instar brains, with anterior to the top and lateral to the right. Open arrowheads mark the boundary between the neuroepithelium (to the right) and the neuroblast zone (to the left).

junction localization (Figure 2b–d) and were positioned at the site of the previously described optic lobe neuroblasts [18,19].

This neuroblast population is closely associated with strings of smaller cells that express the GMC markers nuclear Pros and nuclear Asense (Ase) (Figure 2c, d). Lineage analysis, described below, confirmed that these smaller Pros+ cells are neuroblast progeny. Thus, based on molecular markers and morphology, we detected two distinct populations of cells in the developing optic lobe: neuroepithelial cells and neuroblasts. We found no evidence of a population of symmetrically dividing neuroblasts in the optic lobe.

Optic lobe neuroepithelial cells divide symmetrically, whereas neuroblasts divide asymmetrically

To test our conclusion that neuroepithelial cells divide symmetrically and neuroblasts divide asymmetrically, we assesed the localization of cortical polarity proteins in the optic lobe by immunohistochemistry and live imaging. Insc and aPKC localize to the apical cortex of embryonic and larval neuroblasts [28-30], whereas Mira, Pros, and Pon-GFP are basally localized in some epithelial cells and in all neuroblasts [27,31-34]. We found that Dpn-positive optic lobe neuroblasts always segregate Insc (Figure 2f) and aPKC (data not shown) into the larger neuroblast and Mira, Pros, and Pon-GFP into the smaller GMC (n = 37; (Figures 2d, e, 3c–e). In contrast, most Dpn-negative neuroepithelial cells partition Pon-GFP equally to both

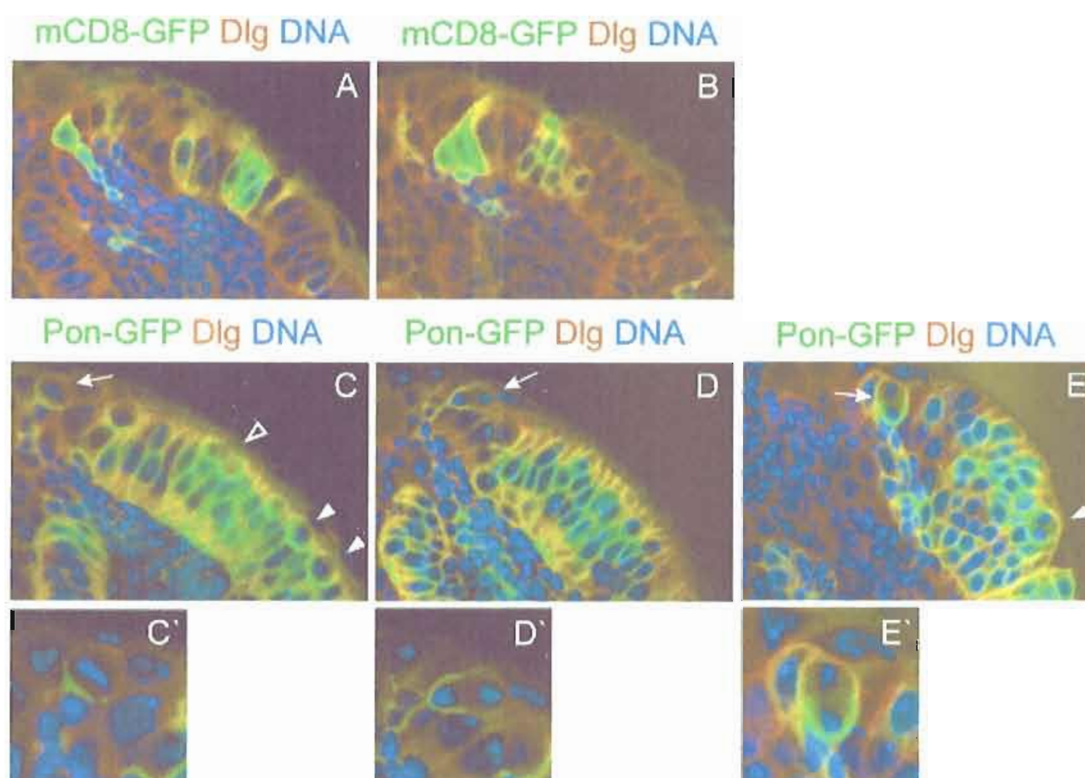

Figure 3. Proliferative symmetric and differentiative asymmetric division depends on the medio-lateral location within the optic lobe. **(a, b)** mCD8-GFP MARCM clones (green) are shown in mid third instar brains. Dlg is in red and DNA in blue. **(a)** A lateral clone contains columnar shaped epithelial cells that presumably were generated by proliferative symmetric divisions (the single confocal section shows three epithelial cells). The clone located at the medial edge of the optic lobe contains neuroblasts with attached strings of progeny cells (the single confocal section shows one neuroblast and three progeny cells). **(b)** A clone at the medial edge of the optic lobe comprises four progenitor cells and one progeny cell (the single confocal section shows two progenitor cells and one progeny cell). **(c-e)** $GAL4^{c855a}$ driven *UAS-pon-gfp* (green) reveals the division mode of optic lobe neuroepithelial cells and neuroblasts. Dlg is in red and DNA in blue. Brains at mid-third (c, d) and early third (e) instar. **(c)** Neuroepithelial cells undergoing mitosis round up at the apical surface of the epithelium and show basolateral Pon-GFP (metaphase: filled arrowheads). Upon cytokinesis Pon-GFP is partitioned equally to both daughter cells (telophase: open arrowhead). At the medial edge of the epithelium optic lobe neuroblasts reveal a basal crescent of Pon-GFP at metaphase (arrow; enlarged in (c')). **(d)** At the medial edge of the epithelium a neuroblast in anaphase segregates Pon-GFP asymmetrically to the basal daughter cell (arrow; enlarged in (d')). **(e)** A more dorsal confocal section reveals a neuroepithelial cell in anaphase segregating Pon-GFP symmetrically to both daughter cells (arrowhead) and a neuroblast (arrow; enlarged in (e')) in anaphase segregating Pon-GFP to the basal daughter cell. All images are single confocal sections, with anterior on top and lateral to the right.

daughter cells (n = 28) (Figure 3c, e) and we did not detect expression of Insc, Mira or Pros. The only exception is a population of Dpn-negative epithelial cells that lie adjacent to the Dpn-positive neuroblasts, which segregate Pon-GFP asymmetrically. These cells are likely to be newly formed neuroblasts with Dpn levels below our detection threshold.

To further characterize the neuroepithelial and neuroblast populations in the optic lobe, we next investigated their cell division patterns in wild-type brains. We used the MARCM system [35] to induce small mCD8-GFP labeled wild-type clones at late second/early third larval instar (48 hours ALH) and analyzed the brains at mid-third instar (72 hours ALH) (Figure 3a). We observed small clones containing two to eight cells with columnar epithelial morphology (n = 7) within the lateral optic lobe, consistent with the expansion of one progenitor via symmetric cell division (Figure 3a). We also saw clones in the medial optic lobe (where the neuroblasts are located) that had one or more large round cells adjacent to a cluster of smaller round cells (n = 11), consistent with neuroblasts dividing asymmetrically to generate a chain of smaller GMCs/neurons (Figure 3a). We conclude that neuroepithelial cells divide symmetrically to generate two neuroepithelial cells, whereas neuroblasts divide asymmetrically to generate smaller progeny.

The combination of our molecular, morphological, and live imaging data allows us to conclude that there are two distinct cell types in the optic lobe. Neuroepithelial cells are found in the lateral region and have a classic columnar epithelial morphology, epithelial molecular markers and epithelial junctions. They undergo symmetric cell division to expand the neural stem cell population. Neuroblasts are found in the medial

region and have a rounded shape and lack epithelial junctions. They divide asymmetrically to self-renew and produce a smaller differentiating daughter cell.

Optic lobe neuroepithelial cells are the progenitors of optic lobe neuroblasts

We next wished to test directly the hypothesis that optic lobe epithelia give rise to optic lobe neuroblasts [17-19]. We performed a clonal analysis using the FLP/FRT system [36] and adjusted clone induction frequency to 1.2 clones per optic lobe. We induced clones expressing a nuclear ß-galactosidase (ß-gal) reporter protein at early second instar (31 hours ALH), when the optic lobe consists primarily of neuroepithelial cells (Figure 1), and assayed the developing clones for cell fate markers at 48 hours or 96 hours ALH. Brains were labeled for ß-gal to show all cells within a clone; for Scrib to outline cell morphology and label epithelial septate junctions; and for Dpn to mark neuroblasts (Figure 4). We observed four classes of clones: neuroepithelial cells only (Figure 4a); neuroblasts and their neuronal progeny only (Figure 4c); neuronal progeny only (data not shown); and mixed clones of neuroepithelial cells, neuroblasts and progeny (Figure 4b). When clones were assayed relatively soon after induction (48 hours ALH), we observed a high percentage of neuroepithelial only clones (22/28), with few neuroblast only clones (5/28) or mixed clones (1/28). In contrast, allowing the clones to develop longer (96 hours ALH) resulted in a majority of the clones being neuroblast/progeny only (20/33) or neuronal progeny only (4/33), with few neuroepithelial only clones (5/33) or mixed clones (4/33)

One example of a clone that supports the idea of a switch from a neuroepithelial to neuroblast cell type is shown in Figure 3b. This clone, at the medial edge of the epithelium, contains four neural progenitor cells and one progeny cell, suggesting that a neuroepithelial cell underwent two rounds of symmetric division to generate four cells; one of these cells then switched to a neuroblast fate and divided asymmetrically, self-renewing and producing a single GMC. Another such clone consisted of 20 neuroepithelial cells, four large round Dpn positive cells, and two smaller round cells (data not shown). We interpret this clone as deriving from a neuroepithelial cell that divided symmetrically to generate 24 cells, four of which switched to a neuroblast fate. Two of these neuroblasts then divided asymmetrically to produce a single GMC each.

Two conclusions can be drawn from our lineage experiments. First, neuroepithelial cells give rise to neuroblasts; initially most clones consist exclusively of neuroepithelial cells but with time most clones contain neuroblasts and their progeny. It is likely that neuroepithelial clones that expand towards the medial edge of the epithelium become partially or completely transformed into neuroblasts. This is consistent both with previous studies and our own observations that the epithelial population shrinks as the neuroblast population expands (Figure 1) [18,19]. Second, at least some neuroblasts ultimately differentiate or die, resulting in clones that consist entirely of neuronal progeny.

Inducing vertical spindle orientation in neuroepithelial cells does not promote neuroblast or neuronal specification.

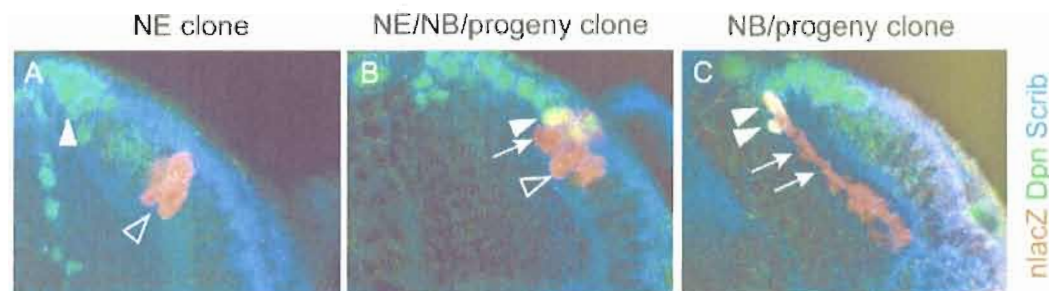

Figure 4. Optic lobe neuroblasts derive from the neuroepithelium in a medial transition zone. **(a-c)** Single FLP-out clones expressing nuclear β-gal (red) in the optic lobe at late third instar (96 hours ALH). Dpn is in green, Scrib in blue. **(a)** An epi only clone containing Dpn negative epithelial cells (marked with β-gal in red, open arrowhead) but no Dpn positive neuroblasts (green, arrowhead). **(b)** An epi/NBs/progeny clone containing Dpn negative epithelial cells (open arrowhead), Dpn positive neuroblasts (arrowhead, yellow) and progeny cells (arrow). **(c)** A NBs/progeny clone containing Dpn positive neuroblasts (arrowheads, yellow) and progeny cells (arrow).

It has been proposed that mammalian neuroepithelial cells, retinal progenitor cells and epidermal stem cells expand their stem cell population by 'horizontal' divisions in which the mitotic spindle aligns perpendicular to the apical/basal axis of cell polarity. They then switch to a 'vertical' division axis to divide asymmetrically and generate novel cell types [2-5,37,38]. It is not known whether a change in cell fate is required to switch the cell division axis (for example, to a cell fate that expresses a protein that modifies spindle orientation), or whether a stochastic change in spindle orientation can lead to a cell fate change (for example, due to the asymmetric partitioning of cell fate determinants). The *Drosophila* optic lobe neuroepithelium represents an excellent model system to determine whether a change in spindle orientation induces new cell fates, or whether a change in cell fate is required to alter spindle orientation.

To switch spindle orientation in neuroepithelial cells we misexpressed Insc in neuroepithelial cells. Expression of Insc in embryonic epithelial cells has been shown to reorient their mitotic spindles from horizontal (perpendicular to the apicobasal axis) to vertical (aligned with the apicobasal axis) [30]. Embryonic Insc misexpression does not lead to obvious changes in the embryonic neuroectoderm. However, not all neuroectodermal cells give rise to neural precursors; most give rise to epidermis. In the optic lobe, all neuroepithelial cells express the proneural gene *sc* and are, therefore, competent to become neuroblasts. Therefore, we investigated whether spindle reorientation can induce a neuroblast fate in this system. In control optic lobe neuroepithelia we detected no Insc protein and the majority of metaphase spindles were aligned horizontally, positioned to give a symmetric cell division (Figure 5a, c). When

Insc is misexpressed within the optic lobe neuroepithelium, the protein localizes apically and the majority of metaphase spindles orients vertically, along the apicobasal axis, positioned to enable an asymmetric cell division (Figure 5b, d). Despite this striking reorientation of the mitotic spindle, we saw no evidence for the induction of ectopic Dpn+ neuroblasts, GMCs, or neurons in the optic lobe (data not shown). We conclude that forcing vertical spindle orientation in neuroepithelial cells is not sufficient to induce neuroblast or GMC cell fates. After Insc misexpression the neuroepithelium is virtually indistinguishable from a control neuroepithelium throughout larval development. We conclude that the resulting apical and basal daughter cells are reintegrated into the epithelium and are only able to switch to a neuroblast fate when they reach the edge of the optic lobe. Thus, the transition from neuroepithelial cell to neuroblast must be due to a cell fate transition that is not regulated by a switch in spindle orientation. We propose that the switch from a neuroepithelial cell to a neuroblast entails the coordinate regulation of multiple downstream events that include the disassembly of epithelial junctions and the transcription of genes that promote vertical spindle orientation.

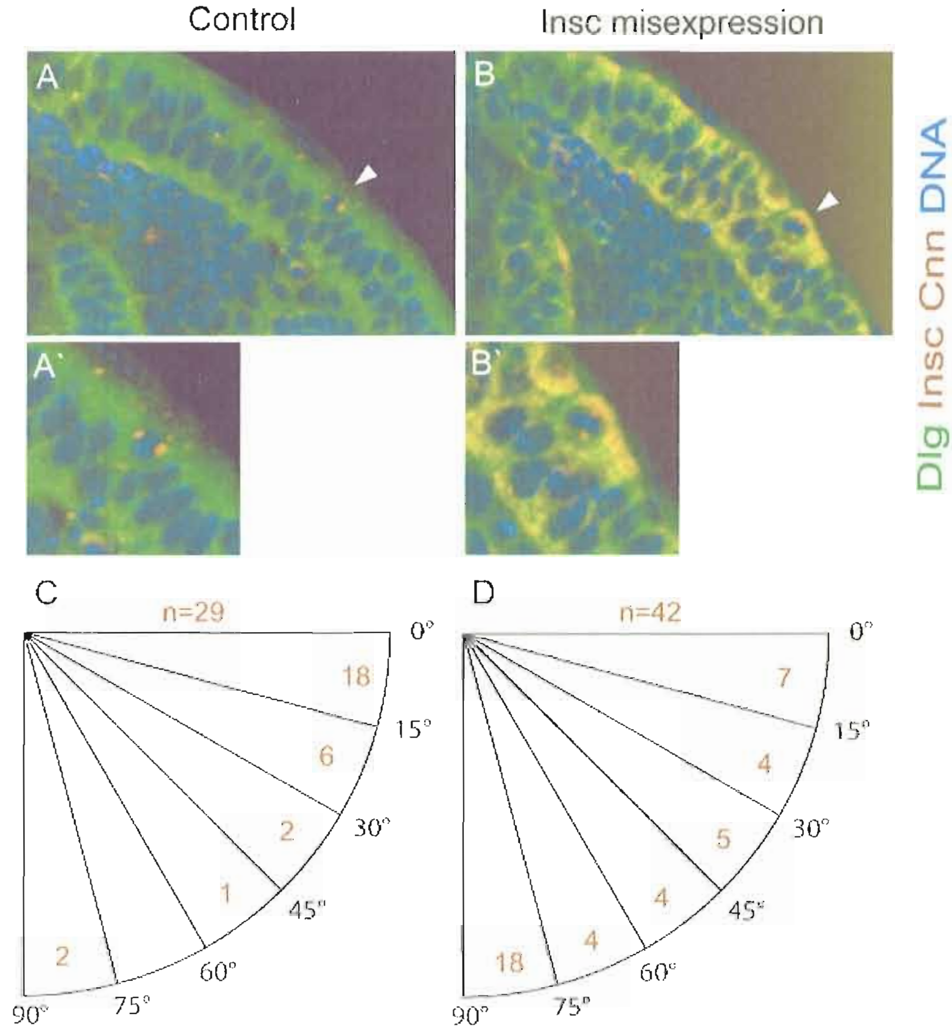

Figure 5. Misexpression of Insc in neuroepithelial cells can induce vertical spindle orientation. Spindle orientation at prometaphase/metaphase was analyzed in neuroepithelial cells at mid third instar (72 hours ALH). (**a**) In control brains the great majority of neuroepithelial cells have a horizontal spindle axis (arrowhead, enlarged in (a')) (n = 29). Note that neuroepithelial cells do not express Insc. (**b**)$GAL4^{c855a}$ driven *UAS-insc* results in apical Insc in neuroepithelial cells and forces spindles into a vertical orientation (n = 42). (**c, d**) Spindle orientation in control optic lobes (c) and optic lobes misexpressing Insc (d). A horizontal spindle axis is 0°; a vertical spindle axis is 90°. The number of neuroepithelial cells is shown in red within six 15° angle sectors from 0° to 90°.

DISCUSSION

In this study we show that optic lobe neuroepithelial cells can be distinguished from optic lobe neuroblast cells by morphology, gene expression and division mode (Figure 6). Neuroepithelial cells occupy the lateral region of the optic lobe and divide in a proliferative symmetric division mode, which expands the neural stem cell pool at an early phase of optic lobe development. At a later stage, progressively more stem cells round up and split off from the medial part of the optic lobe epithelium. These optic lobe neuroblasts lose their adherens junctions and start to divide asymmetrically, generating smaller GMCs towards the growing medulla cortex.

The optic lobe neuroepithelium is similar to the embryonic ventral neuroectoderm in that it expresses the same junctional complexes and the proneural gene *scute*. Optic lobe neuroblasts exhibit an apicobasal polarity and express pan-neural genes such as *dpn* and *ase*. However, most embryonic neuroectodermal cells adopt an epidermal fate, whereas optic lobe epithelial cells eventually give rise to neuronal and glial cells (hence it is a neuroepithelium). Recently, it has been suggested that embryonic neuroblasts require an extrinsic signal, provided by the overlying epithelium, to coordinate their division axis with apicobasal tissue polarity [39]. As optic lobe neuroblasts do not delaminate from an overlying (apical) epithelium, but rather segregate laterally from the adjacent neuroepithelium, they do not maintain contact with an overlying epithelium.

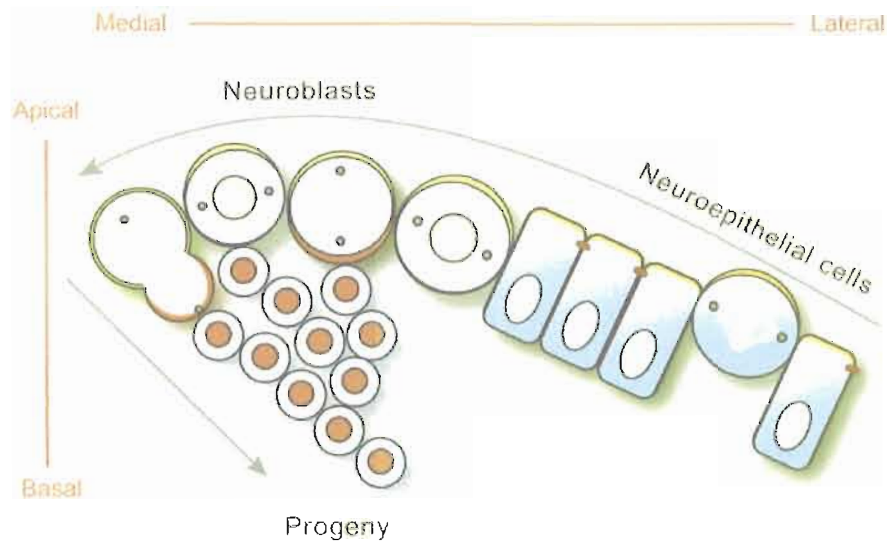

Figure 6. Model of neuroepithelial to neuroblast transition at the medial edge of the optic lobe. At the medial edge of the optic lobe columnar neuroepithelial cells disassemble adherens junctions and undergo a transition to neuroblasts. Neuroepithelial cells divide symmetrically with horizontal spindle orientation, which results in the expansion of the progenitor pool. Medial neuroblasts divide asymmetrically with vertical spindle orientation and bud off smaller ganglion mother cells (GCMs) towards the presumptive medulla cortex.

Nonetheless, they are still able to reorient their mitotic spindles and divide asymmetrically along the apicobasal axis, budding off GMCs towards the developing medulla cortex. The cortex glial cells, which enwrap the larval brain [40], may provide apicobasal positional information to the optic lobe neuroblasts in place of an overlying epithelium.

In the *Drosophila* embryo the proneural genes *ac*, *sc*, and *lethal of scute* are expressed in the neuroectoderm [41,42], as is the transcription factor Pros and its adaptor Mira [32-34]. Although we saw proneural gene expression in the optic lobe neuroepithelium, we detected neither Pros protein nor Mira mRNA or protein. This contrasts with the embryonic neuroectoderm, where both Pros and Mira are expressed and localize basolaterally, and suggests that the transcriptional cascade underlying optic lobe neuroblast formation is different from embryonic neuroblast formation. In the optic lobe, Mira and Pros are first expressed in neuroblasts. Here they localize in a crescent at the basal cortex and segregate into the medulla GMCs (Figure 2d, e) (in contradiction to an earlier study suggesting that Pros is not expressed in optic lobe neuroblasts and GMCs, but only in mature neurons [16]).

Possible mechanisms for the transition from optic lobe neuroepithelial cells to neuroblasts

Our clonal analysis demonstrates that optic lobe neuroblasts derive from the optic lobe neuroepithelium in a temporally and spatially regulated fashion. In assessing the clonal relationship between optic lobe neuroepithelial cells and neuroblasts we recovered

only a small number of mixed clones containing both epithelial cells and neuroblasts. Instead, most clones contained either only epithelial cells or neuroblasts and their progeny. The transition from a neuroepithelium to neuroblasts could occur by a neuroepithelial cell dividing symmetrically, generating two neuroblasts, or by a neuroepithelial cell dividing asymmetrically, generating one neuroepithelial cell and one neuroblast. Our clonal analysis does not distinguish whether one or both of these mechanisms occur.

A mediolateral gradient of a morphogen may regulate the changes in gene expression required to induce the neuroblast fate. Once the neuroepithelium has proliferated to reach a critical size, the most medial cells would be pushed beyond the range of the morphogen's activity, and would be induced to become neuroblasts. A possible candidate for this morphogen is Decapentaplegic (Dpp), the *Drosophila* BMP2/4 homologue, which shows regional, Wingless-dependent, expression in the optic lobe [43]. Mutations in either *wg* or *dpp* lead to a reduction in the size of the optic lobe and to defects in the optic lobe neuropile and it has been suggested that these defects might be caused by failure in progenitor specification in the developing optic lobe [43].

Similarities to vertebrate neural stem cells

The transition of optic lobe neuroepithelial cells to neuroblasts in the optic lobe is reminiscent of the transition of neuroepithelial cells to radial glia in the developing vertebrate neocortex and in the neural tube. Mammalian neuroepithelial cells, or neural stem cells, first undergo symmetric division to expand the neural stem cell pool. This is

followed by self-renewing, asymmetric division, during which neuroepithelial cells down-regulate epithelial features such as tight junctions (but not adherens junctions) and self renew while also generating cells with a more restricted developmental potential [44-50].

The organization of the optic lobe also bears comparison with the vertebrate retina, where a spatially ordered structure is evident with respect to both cellular development and differentiation: in the ciliary marginal zone (CMZ) the youngest and least determined stem cells are closest to the periphery, the proliferative retinoblasts are medial and the cells that have stopped dividing are at the central edge [51,52]. Similarly in the optic lobe, the neuroepithelial cells are found laterally, the neuroblasts medially, and the ganglion cells towards the inside of the lobe.

The striking similarities between the optic lobe and the CMZ suggest that similar genetic pathways may be involved in both systems. Recently, it was shown that Insc, which regulates spindle orientation in *Drosophila* neuroblasts, is also expressed in the vertebrate retina [5]. Insc expression in embryonic neuroblasts and optic lobe neuroblasts is one of the earliest signs of neuroblast specification; neither the embryonic ventral neuroectoderm nor the optic lobe neuroepithelium express *insc*. Interestingly, whereas *insc* is expressed in vertically dividing neuroblasts in the *Drosophila* optic lobe and embryonic central nervous system, in the mammalian retina it is expressed in both vertically dividing cells (where it localizes apically) and horizontally dividing cells (where it is apicolateral). This suggests that, in the vertebrate retina, the division plane is determined by whatever localizes Insc, rather than solely by the presence of Insc.

Zigman *et al.* [5] show that reducing the levels of Insc increases the number of horizontal divisions at the expense of vertical divisions. This leads eventually to a decrease in the number of early differentiating photoreceptor cells and eventually to an increase in later differentiating bipolar neurons. From these results the authors infer that a switch from vertical to horizontal division increases the stem cell pool at the expense of early differentiated neurons, that is, that spindle orientation determines the fate of the progenitor cells.

CONCLUSION

Here we show that the optic lobe harbors two neural stem cell types: neuroepithelial cells, which divide symmetrically to expand the neural stem cell pool, and neuroblasts, which divide asymmetrically to self-renew and generate differentiating GMCs. Neuroblasts derive from the neuroepithelium in a developmentally and spatially regulated fashion. Reorientation of the mitotic spindle in *Drosophila* neuroepithelial cells, as directed by ectopic expression of Insc, is not sufficient in and of itself to induce the neuroblast fate and does not lead to premature neurogenesis. Instead, spindle orientation responds to cell fate rather than promoting it. Cell fate specification in neuroblasts leads to expression of *insc* and spindle reorientation. A second consequence of neuroblast fate specification is the expression of Pros and Mira. Thus, when the spindle reorients in the neuroblast, cell division generates two different cell types due to the asymmetric partitioning of Pros. In the optic lobe the different division planes of neuroepithelial cells and neuroblasts lead to stratified layers of cells that contribute to the morphogenesis of the brain lobes (Figure 6). Thus, one key role of regulated spindle

orientation in the optic lobe may be in positioning cells appropriately within the tissue, a function similar to what has been proposed for mammalian skin [3].

MATERIAL AND METHODS

Fly strains

Flies were raised on cornmeal medium at 25°C. Oregon R and *yw* were used as control strains. To assay *sc* expression the *3.7sc-lacZ* line [53] (from P Simpson, Cambridge, UK) was used. The following driver and responder lines were used: *GAL4*^{c855a} [20,21] (from the Bloomington *Drosophila* Stock Centre, Bloomington, Indiana, USA), *UAS-pon-gfp* [31], *UAS-pon-gfp; UAS-H2B-mRFP1* [54] (from Y Bellaiche, Paris, France) and *UAS-insc/TM3* [30] (from J Knoblich, Vienna, Austria). For MARCM clones we used *hs-Flp; FRT40A, tub-Gal80; tub-Gal4/TM6B* and *FRT40A; UAS-mCD8-GFP, UAS-nlslacZ* [8] (from B Bello, Basel, Switzerland). For flip-out clones and lineage tracing *hs-FLP(f38)* and *act5C(FRT)nlslacZ* (from Bloomington) were used.

Staging of larvae and clone induction

Freshly hatched larvae were collected in a 4 to 6 hour time window and staged on cornmeal medium to late first/early second instar (21 to 27 hours ALH; after hatching), late second/early third instar (45 to 51 hours ALH), mid third instar (69 to 75 hours ALH) or late third instar (93 to 99 hours ALH). Targeted gene expression was achieved with the GAL4/UAS system. The *GAL4*^{c855a} line drives targeted gene expression in all optic lobe progenitor cells from first instar onwards. For MARCM experiments clones were induced by heat shock for 30 minutes at 37°C at late second/early third instar with the following

genotype: *yw, hs-FLP; FRT40A, +/FRT40A, tub-GAL80; UAS-mCD8:GFP, UAS-nlslacZ/tub-GAL4*. Larvae were dissected and fixed at mid third instar for clone examination. For flip-out clonal analysis clones were induced by heat-shock for 45 minutes at 37°C at 31 hours ALH. Clones were examined at 48 hours or 96 hours ALH.

Insc misexpression and analysis of spindle axis

For insc misexpression $GAL4^{c855a}$ was crossed to UAS-insc/TM3. The spindle axis was analyzed in $GAL4^{c855a}$*/UAS-insc* and $GAL4^{c855a}$*/TM3* control brains. For cells in prometaphase and metaphase a line was drawn joining the two centrosomes. The angle of the spindle axis was calculated in reference to the tangent at the neuroepithelial surface. We only considered Dpn negative cells that were within the neuroepithelium and not neighboring Dpn positive neuroblast regions.

Immunocytochemistry and image acquisition

Larval tissues were fixed and immunostained as previously decribed in [55]. Primary antibodies used in this study include rabbit anti-Scrib 1:2500 [56], rat anti-DE-Cad 1:100 (Serotec, Raleigh, North Carolina, USA), rabbit anti-PatJ 1:1000 [57] (renamed PatJ [58]), mouse anti-Dlg 4F3 1:100 (Developmental Studies Hybridoma Bank (DSHB), Iowa City, Iowa, USA), rat anti-Dpn 1:2 [10], rabbit anti-Ase 1:500 (from A Jarman, Edingburgh, UK), mouse anti-Pros MR1A (DHSB) 1:30, rabbit anti-Mira A96c 1:1000 [33] (from YN Jan, San Francisco, USA), rabbit anti-Insc 1:500 (from W Chia, Singapore, Singapore) mouse anti-ßGAL 1:500 (Promega, Madison, Wisconsin, USA), rabbit anti betaGal 1:10000 (Cappel, Organon Teknika Corporation, West Chester, Pennsylvania, USA), rabbit anti-Cnn 1:1000 (unpublished, kindly provided by J Raff,

Cambridge, UK), rabbit anti-GFP 1:1000 (Abcam, Cambridge, Cambridgeshire, UK), and chicken anti-GFP 1:20 (Upstate, Charlottesville, Virginia, USA). DNA was stained with DAPI (Sigma-Aldrich, Gillingham, Dorset, UK). Fluorescent conjugated secondary antibodies Alexa405, Alexa488, Alexa568, Alexa633 were used (Molecular Probes, Invitrogen, Paisley, Renfrewshire, UK). Images were acquired with a Leica SP2 confocal microscope and processed with Imaris 3.2 (Bitplane, Zurich, Switzerland) and Adobe Photoshop 8.0. Figures and illustrations were made using Adobe Illustrator 11.0.

Live imaging

Larval brains expressing $GAL4^{c855a}$ driving Pon-GFP and H2B-mRFP1 were dissected at third instar and placed on poly-Lysine (0.002%) coated coverslips in a chamber containing fat body conditioned D22 insect medium, 7.5% bovine calf serum [59]. Cell divisions were imaged using a Zeiss Meta510 inverted confocal microscope equipped with a 40 × NA 1.4 oil-immersion objective.

Fluorescent in situ hybridization

Probes were made by using PCR amplification from a cDNA library with the reverse primer containing a T7 polymerase promoter, CAGTAATACGACTCACTATTA. PCR was performed using Phusion Taq (New England Biolabs, Hitchin, Hertfordshire, UK) with the following cycles: 98°C for 2 minutes; 5 times (98°C for 20 s, 50°C for 20 s, 72°C for 1 minute); 35 times (98°C for 20 s, 59°C for 20 s, 72°C for 2 minutes); and 72°C for 5 minutes. The primers were designed using Primer3 [60] with an optimum length of 24 base-pairs (bp) and optimum melting temperature (Tm) of 60°C. UTP-Dig (Roche Diagnostics, Burgess Hill, West Sussex, UK) labeled RNA probes were generated

from template PCR products by *in vitro* transcription. For better tissue penetration the probes were degraded to an average size of 500 bp fragments using a carbonate fragmentation buffer [61]. Fluorescent *in situ* hybridization (FISH) was performed according to [62] with minor modifications. Larval brains were fixed in 4% paraformaldehyde in phosphate-buffered saline for 20 minutes. Hybridization was performed at 65°C for 12 to 16 hours. Fluorescent signal was obtain by using a Tyramide Amplification Kit (Molecular Probes, Invitrogen). Primers for probes were: *ac*_forward, GAAAATCACTCTGTTTTCAACGAC; *ac*_reverse, **CAGTAATACGACTCACTATTA**TCAGTTTAATGTCCTCAATGTATGC; *sc*_forward, ACAACGAAAAGCACTACCATGTCA; *sc*_reverse, **CAGTAATACGACTCACTATTA**AGAAAATAGGGCGTGGTGGTAAAT; *mira*_forward, GGTAGAGAATCTCCAGAAGACCAA; *mira*_reverse, **CAGTAATACGACTCACTATTA**AAACGCGAAAGATAGAAAACAATC. The nucleotides in bold represent the T7 polymerase binding site.

Authors' Contribution

JQB carried out the FLP-out clonal analysis, participated in the expression study, participated in designing the Insc missexpression study and helped in drafting the manuscript. BE participated in the expression studies, carried out the Pon-GFP live study, the MARCM study, performed the Insc misexpression study and helped drafting the manuscript. NRS participated in designing and performing the Insc misexpression study. AHB and CQD participated in the design and coordination of the study and drafted the final manuscript. All authors read and approved the final manuscript.

Bridge to Chapter III

By utilizing a clonal analysis to find lineages relationships in the optic lobe, I was able to gain a further understanding of the lineages relationships within other central brain regions. Following this approach I found that there are novel populations of central brain neuroblasts that behave differently than other previous characterized central brain neuroblast lineages. Using my previously published data I show that the previously uncharacterized central brain neuroblast lineages contain transit amplifying ganglion mother cells. Further, I show that the parent neuroblast does not asymmetrically segregate the fate determinant Prospero thus producing a ganglion mother cell that has limited differentiation potential and reverts to a neuroblast like fate. Importantly I show that these proliferative ganglion mother cells can generate up to 10 neurons, something never observed in other central brain neuroblast lineages.

CHAPTER III

IDENTIFICATION OF *DROSOPHILA* TYPE II NEUROBLAST LINEAGES CONTAINING TRANSIT AMPLIFYING GANGLION MOTHER CELLS

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INTRODUCTION

In many mammalian tissues, stem cells generate lineage-restricted ''transit amplifying cells'' that can proliferate to expand the number of differentiated progeny made from a single precursor (Morrison and Kimble, 2006; Nakagawa et al., 2007). Teasing out the mechanisms that regulate stem cell proliferation and self-renewal from those regulating proliferation of transit amplifying progenitors is an important goal of stem cell biology, and has been complicated by the difficulty in identifying each type of progenitor in vivo or in vitro.

The *Drosophila* CNS develops from neural precursors called neuroblasts, which have recently become a model for studying neural stem cell self-renewal (Bello et al., 2006; Betschinger et al., 2006; Lee et al., 2006a,b,c; Wang et al., 2006) (reviewed in Doe, 2008). Neuroblasts divide asymmetrically in cell size and fate to form a larger neuroblast and a smaller ganglion mother cell (GMC). The neuroblast

continues to proliferate, whereas the GMC typically generates just two post-mitotic neurons (Goodman and Doe, 1993; Lee and Luo, 1999; Pearson and Doe, 2003). Many proteins are asymmetrically segregated during neuroblast mitosis: the apical proteins Bazooka, aPKC, Par-6, Partner of Inscuteable (Pins), and Inscuteable (Insc) are segregated into the neuroblast, whereas the basal proteins Numb, Miranda (Mira), Prospero (Pros), and Brain tumor (Brat) are localized into the GMC (reviewed in Caussinus and Hirth, 2007). aPKC promotes neuroblast self-renewal, whereas the basal proteins Numb, Mira, Brat, and Pros all act to inhibit self-renewal and promote neuronal differentiation (Bello et al., 2006; Betschinger et al., 2006; Choksi et al., 2006; Lee et al., 2006a,c; Wang et al., 2006). Neuroblast transcription factors include the basic-helix-loop-helix protein Deadpan (Dpn), which promotes optic lobe proliferation (Wallace et al., 2000), but has not been assayed for a role in neuroblast proliferation. In contrast, the Pros transcriptional repressor is nuclear in GMCs and young neurons (Hirata et al., 1995; Knoblich et al., 1995; Spana and Doe, 1995; Li and Vaessin, 2000), where it downregulates cell cycle gene expression to restrict GMCs to one terminal mitosis (Hirata et al., 1995; Knoblich et al., 1995; Spana and Doe, 1995; Li and Vaessin, 2000).

Here, we identify a novel ''Type II'' neuroblast lineage that contains transit amplifying GMCs (TA-GMCs) that can each generate up to 10 neurons. These neuroblast lineages provide a model system for studying the similarities and differences between transit amplifying neural progenitors in Drosophila and mammals, and may help explain the phenotypic variation previously observed in wild type and mutant Drosophila larval

brains. While this article was in review, similar reports were published (Bello et al., 2008; Bowman et al., 2008), and our data are consistent with these studies.

MATERIALS AND METHODS

Fly stocks and clonal analysis

To generate mosaic analysis with repressible cell marker (MARCM) clones we crossed *hs-flp ;tubP-gal80, FRT40A / CyO ; tubP-gal4, UAS-mcd8::GFP / TM6 Tb* to *FRT40A (ovoD) / CyO* and assayed clones in progeny of the genotype *hs-flp ; tubP-gal80, FRT40A / FRT40A (ovoD) ; tubP-gal4, UAS-mcd8::GFP / +*. We picked first or second instar larvae by morphology and incubated them at 37oC for 25-30 min, aged them for 48h, and then dissected, fixed, and stained the brains (see below). This protocol generates a low frequency of clones per brain lobe; any brain lobe containing clones that could not be individually identified was discarded.

Immunostaining and confocal analysis

Larval brains were dissected in Schneider's medium (Sigma, St. Louis, MO); fixed in 100 mM Pipes (pH 6.9), 1 mM EGTA, 0.3% Triton X-100, and 1 mM MgSO4 containing 4% formaldehyde for 25 min; washed 30 min in phosphate buffered saline (PBS); washed 30 min in PBS containing 0.3% Triton X-100 (PBS-BT); and incubated with primary antibodies in PBS-BT overnight at 4°C. Primary antibodies were rat Dpn monoclonal (1:1), rabbit phosphohistone H3 (1:1000; Upstate, Billerica, MA), mouse Pros monoclonal (purified MR1A, 1:1000), rabbit GFP (1:1000; Sigma, St. Louis, MO), rabbit Pins (1:1000), guinea pig Mira (1:500), mouse BrdU (1:50; Sigma, St. Louis, MO),

mouse Fasciclin II (1:100; Developmental Studies Hybridoma Bank, DSHB), and rat Elav (1:10; DSHB). Secondary antibodies were from Molecular Probes (Eugene, OR). Antibodies without named sources were made in the laboratory; details are available on request. Images were captured with a Biorad Radiance or Leica SP2 confocal microscope and processed in Photoshop 7 (Adobe, San Jose, CA). Three-dimensional brain reconstructions, mushroom body iso-surface representations, and movies were generated using Imaris software (Bitplane, Zurich, Switzerland).

BrdU pulse/chase experiments

Bromodeoxyuridine (BrdU) was purchased from Roche (Basel, Switzerland), dissolved in 1:1 DMSO:acetone, and mixed with food media at a final concentration of 1 mg/mL. Larvae were fed on BrdU-containing food for 4.5 h and immediately fixed for pulse experiments, or allowed to develop on food lacking BrdU for 18h before fixation for chase experiments. Larval brains were dissected, fixed, and antibody stained as described above with the addition of a 2N HCl treatment for 30 min prior to BrdU staining.

Identifying type I and type II neuroblast and GMC lineages

Clonal analysis (Figure 1). Type I neuroblast clones were uniquely identified by the presence of one large (>8μm diameter) neuroblast containing nuclear Dpn and cytoplasmic Pros (Dpn+ $Pros^{cyto}$) together with many small (<5μm diameter) progeny that lacked Dpn and had nuclear Pros (Dpn- $Pros^{nucl}$). Cells furthest from the neuroblast were Dpn- Pros- mature neurons that extended GFP+ axons into the brain. Type I GMC clones were identified by lack of a large neuroblast, and were assayed only in the dorsoanterior lateral (DAL) region, where no type II neuroblasts exist (see Figure 2). Type I GMC

clones never had more than two cells. Type II neuroblast clones were identified by the presence of one large Dpn+ Pros^{cyto} neuroblast with the unique and defining feature that the clone also contained many small (<5μm diameter) Dpn+ Pros^{cyto} cells. Cells furthest from the neuroblast were Dpn- Pros- mature neurons that extended GFP+ axons into the brain. Type II TA-GMCs clones were identified by (i) their lack of a large neuroblast; (ii) their ability to make >2 progeny, which is never observed in type I GMC clones; and (iii) the presence of small (<5μm diameter) Dpn+ Pros^{cyto} cells, which are never observed in type I lineages. We observed one and two cell clones in all regions of the brain; we assume they are made by both type I and type II lineages.

Whole brain analysis (antibody stains and BrdU experiments).

Type I neuroblasts can be uniquely identified as a large Dpn+ or Mira+ cell (>8μm diameter) contacting only small (<5μm diameter) Pros^{nucl} cells (the GMCs). Type I neuroblasts are found in the DAL region of the brain, where no type II neuroblasts exist, and thus for consistency we restricted our analysis of type I lineages to this brain region. Type II neuroblasts can be identified as a large Dpn+ cell contacting small Dpn+ Pros^{cyto} cells (TA-GMCs) or in BrdU experiments as a Mira+ neuroblast contacting a large group of small Mira+ cells.

Locating type I and type II lineages in the brain.

Central brain regions (i.e. the brain excluding the lateral optic lobes) were identified and named as previously described (Pereanu and Hartenstein, 2006). Briefly, we used Fasciculin II as a positional marker and Dpn as a neuroblast marker; double labeled brains were oriented according to Pereanu and Hartenstein (2006) to determine

neuroblast position relative to the Fasciculin II pattern. In this way, we mapped the approximate location of type I and type II neuroblasts; we found that type I neuroblasts were the sole occupants of the DAL brain region, whereas the type II neuroblasts were located in subsets of the following brain regions: dorsoposterior medial (DPM), dorsoposterior lateral (DPL), dorsoanterior medial (DAM), centromedial (CM), and centroposterior (CP) (yellow patches in Figure 2; Supplemental Table 1).We could individually identify only one type II neuroblast (the DPMpm1 neuroblast; Figure 2C') due to natural variation in neuroblast position (Pereanu and Hartenstein, 2006); relatively few axon projections in the clones; and similarity between closely positioned neuroblasts (Pereanu and Hartenstein, 2006). To minimize regional variation in neuroblast lineages, we restricted our analysis of type I neuroblasts to the DAL region, and type II neuroblasts to the DPM region.

RESULTS

Clonal Analysis Reveals two Types of Brain Neuroblast Lineages

During our clonal analysis of a larval neuroblast self-renewal mutant we realized that wild type brains have two distinct types of neuroblast lineages (J.Q.B. and C.Q.D., in preparation). This article describes these two types of lineages. We used mosaic analysis with repressible cell marker (MARCM; Lee and Luo, 1999) to generate GFP-marked single cell clones in the larval brain. Depending on the cell in which chromosomal recombination occurs, it is possible to label a single neuroblast and all its progeny, a

single GMC and all its progeny, or a single neuron derived from a terminal mitosis (Lee and Luo, 1999). We induced a low density of clones randomly throughout the brain at either mid-first or mid-second larval instar and assayed all clones 48 h after induction (see Fig. 1). We find two distinct neuroblast lineages: a ''Type I'' lineage that matches previously reported neuroblast lineages (Goodman and Spitzer, 1979; Lee and Luo, 1999; Pearson and Doe, 2003), and a novel ''Type II'' lineage that is larger and more complex.

Type I Neuroblast Lineages. Type I neuroblast clones always contained one large (>8 um diameter) neuroblast near the surface of the brain that had nuclear Dpn and cytoplasmic Pros (Dpn $Pros^{cyto}$) (100%; n ¼ 26; Fig. 1(A); Supplemental Table 1). These clones always contained a column of smaller cells that lacked Dpn and had nuclear Pros (Dpn-$Pros^{nucl}$), with the occasional presence of a single Dpn+ small cell contacting the neuroblast, which is likely to be a newborn GMC (Supplemental Table 1). The cells furthest from the neuroblast were Dpn- Pros- mature neurons that extend GFP1 axons into the central brain (data not shown). Type I neuroblast lineages are the sole occupants of the dorsoanterior lateral (DAL Pereanu and Hartenstein, 2006) brain region, but can also be found in all other brain regions (see Fig. 2). To minimize regional variation in neuroblast lineages, we restricted our analysis of Type I neuroblasts to the DAL region. Type I GMC clones were assayed only in the DAL region, where no Type II neuroblasts were observed. All clones lacking a large Dpn1 neuroblast were considered to be GMC clones, and these GMC clones generated at most just two cells [100%, n= 9; Fig. 1(B)]. Thus, Type I lineages are identical to those reported for Drosophila embryonic

neuroblasts (Goodman and Doe, 1993; Pearson and Doe, 2003), larval mushroom body neuroblasts (Lee and Luo, 1999), and grasshopper neuroblasts (Goodman and Spitzer, 1979).

Type II Neuroblast Lineages. Type II neuroblast clones always contained one large (>8 um diameter) Dpn+ neuroblast near the surface of the brain, but also contained a distinctive group of small (<5 um diameter) Dpn+ cells that lack nuclear Pros [100%; n =17; Fig. 1(C); Supplemental Table 1]. There are also usually 1–2 small cells in direct contact with the neuroblast that lack both Dpn and nuclear Pros [Fig. 1(C), arrows]. These two types of small cells are never observed in Type I clones and are a defining feature of Type II clones. Type II neuroblast clones are found in several brain regions, including a cluster within the DPM region (Fig. 2, yellow shading).

One Type II neuroblast appears to be the previously identified DPMpm1 neuroblast (Pereanu and Hartenstein, 2006) based on its distinctive axon projection that bifurcates over the medial lobe of the mushroom body before crossing the midline [Fig. 2(C0), inset; Movie 1].

Type II GMC clones were identified by the lack of a large Dpn1 neuroblast. All brain regions that contained Type II neuroblast lineages produced GMC clones of greater than two cells (range, 3–10 neurons; average 4.86; n=25; Fig. 1(D,E); Supplemental Table 1); all brain regions that lacked Type II neuroblast lineages never generated [2 cell GMC clones (see above). Type II GMC clones often contained Dpn+ Pros^{cyto} small cells that are unique to Type II neuroblast lineages [Fig. 1(D); arrowhead], confirming that these clones are sub-lineages of a Type II neuroblast lineage. We conclude that Type II

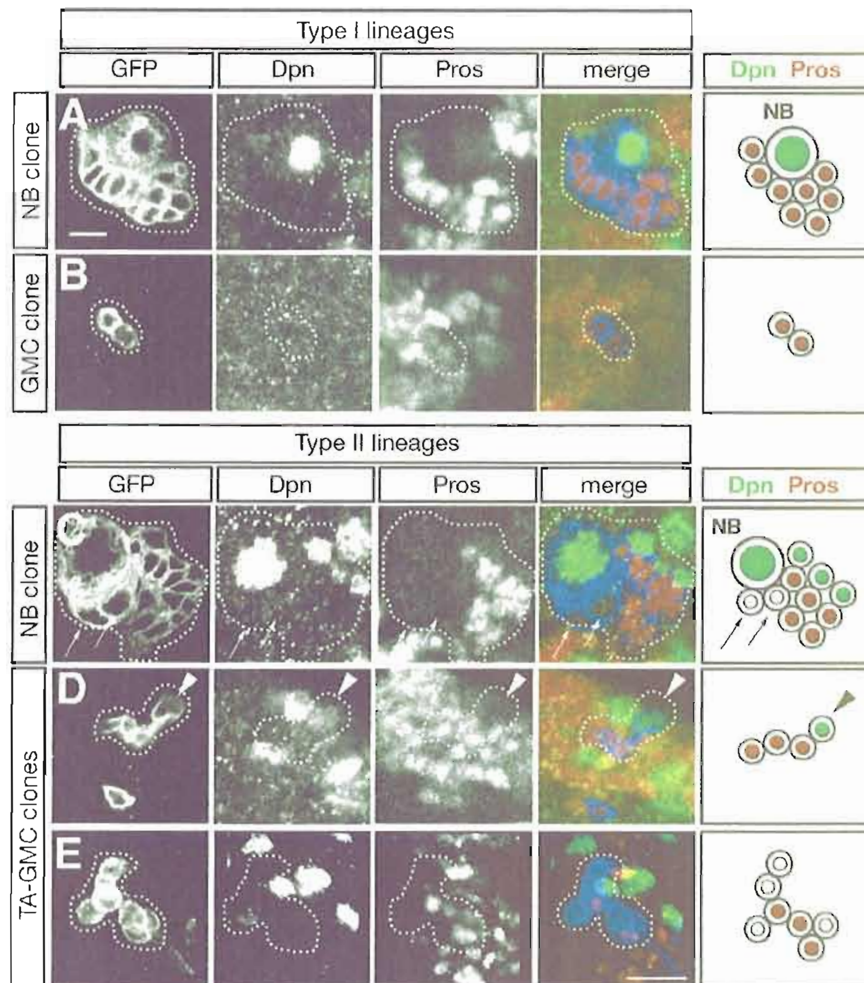

Figure 1 Clonal analysis identifies two types of larval neuroblast lineages. A–E: Neuroblast (NB) and GMC clones stained for Deadpan (Dpn, green), Prospero (Pros, red), and the clone marker GFP (green, outlined). Right panel shows summary of markers: green, nuclear Dpn cytoplasmic Pros (Dpn+ $Pros^{cyto}$); red, Dpn-negative nuclear Pros (Dpn- $Pros^{nucl}$). Type I clones were assayed in the DAL brain region; type II clones were assayed in the DPM brain region. A: Type I neuroblast clone containing one large Dpn+ $Pros^{cyto}$ NB and many Dpn- $Pros^{nucl}$ GMCs. B: Type I GMC clone containing two $Pros^{nucl}$ immature neurons that lack GFP+ axons (data not shown). C: Type II neuroblast clone containing one large Dpn+ $Pros^{cyto}$ NB and smaller progeny including two Dpn- Pros- cells closely-associated with the neuroblast (arrows), several Dpn+ $Pros^{cyto}$ cells, and several Dpn- $Pros^{nucl}$ cells. D: Type II TA-GMC small clone containing one Dpn+ $Pros^{cyto}$ cell (arrowhead) and three Dpn- $Pros^{nucl}$ cells. E: Type II TA-GMC large clone containing several Dpn- $Pros^{nucl}$ cells, and a pool of Dpn- Pros- mature neurons (based on their GFP+ axon projections). Scale bar=6.24 um.

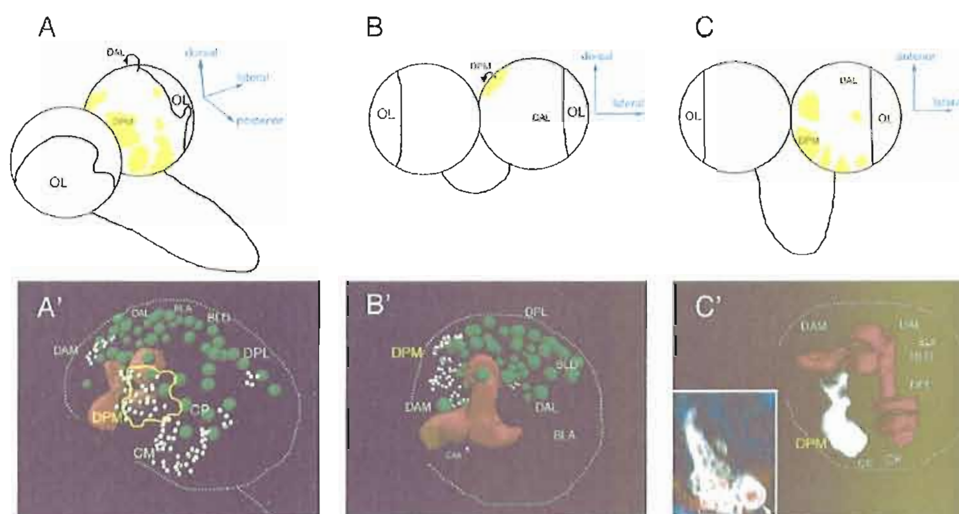

Figure 2 Type I and type II neuroblast lineage locations within the brain. A–C: Schematics of the third instar larval brain showing brain regions according to Pereanu and Hartenstein (2006). Type I neuroblast are found in all brain regions, but only type I neuroblasts are located in the dorsoanterior lateral (DAL) region, which is where we performed all type I lineage assays. Type II neuroblast are found in several brain regions (yellow shading); the largest number are in the dorsoposterior medial (DPM) region. The orientation of each brain is indicated by the axial arrows. OL, optic lobe. (A0–B0) Three-dimensional reconstruction of a confocal image stack of a brain lobe double-label for Dpn (green spheres, neuroblasts; silver spheres, TA-GMCs) and Fasciculin II (mushroom body, red). Orientation is the same as the panel above. This brain lobe is shown in Movie 2. C': Three-dimensional reconstruction of a confocal image stack of a brain lobe containing a type II neuroblast clone (white) stained for Fasciculin II (mushroom body lobes, red). The clone extends medially across the midline but the fine axon processes do not show up in this image; they can be seen in Movie 1. (C' inset) Optical section from brain used to generate the image shown panel C'. Clone marker (GFP, white); Deadpan (red); neuroblast in clone, arrow; TA-GMCs in clone, bracket. OL, optic lobe; DPM, dorsoposterior medial (yellow outline); DAL, dorsoanterior lateral; DAM, dorsoanterior medial; DPL, dorsoposterior lateral; BLP, basolateral posterior; BLA, basolateral anterior; BLD, basolateral dorsal; BA, basoanterior; CP, centroposterior; CM, centromedial. Regions in smaller fonts are towards the back of the lobe. Scale bar= 20 um. Movie 1: Confocal image stack of the brain shown in Figure 2C' inset to illustrate the axon projections of the DPMpm1 neuroblast clone. The medial half of the brain lobe is shown; DPMpm1 clone (white; right-most clone in the brain), Dpn1 neuroblasts and TA-GMCs, red (labeled in the Figure 2C' inset); Fasciculin II+ neuropile, blue. Movie steps from dorsal surface to ventral surface. Movie 2: Rotation of the brain shown in Figure 2A',B'. Large Dpn+ neuroblasts (>8 um, green); small Dpn+ TA-GMCs (<5 um, silver); Fasciculin II+ mushroom body (red). The first frame of the movie is the same orientation as shown in Figure 2B'.

neuroblasts generate GMCs that produce more than two neurons. Because Type II GMC clones could generate several fold more neurons than a Type I GMC, we call them ''transit amplifying GMCs'' or TA-GMCs.

TA-GMC clones also contained small cells with nuclear Pros [Fig. 1(D,E)]; we suggest that these cells are equivalent to Type I GMCs based on their cell division profile (see next section), and because we observed two cell clones in regions of the brain that contained Type II neuroblast lineages. However, we can't rule out the possibility that some of these nuclear Pros cells are post-mitotic immature neurons (see Discussion). If Type II lineages generate TA-GMCs that make an average of twice as many neurons as a Type I lineage, we would expect Type II lineages to generate approximately twice as many neurons over the same time span compared with Type I lineages. Indeed, we find that when Type I or Type II clones are grown for the same length of time (between clone induction and analysis), Type II clones generate approximately twice as many neurons. Type I clones in the DAL generate 40.4 +/-3.1 cells (n=16; clone developing 24–72 h after larval hatching [ALH]), whereas Type II lineages in the DPM generate 71.2 +/- 6.3 cells (n =5; clone developing 24–72 h ALH). In all cases the final Type I and Type II neuroblast clones contained a single large >8 um diameter Dpn+ neuroblast, ensuring that only single neuroblast clones were counted. We conclude that Type II TA-GMCs generate more neurons than Type I GMCs, and that Type II lineages generate more neurons than Type I lineages.

Asymmetric Cell Division within Type I and Type II Lineages

Here, we characterize the cell division patterns within Type I and Type II lineages to help understand the relationship between different cell types in a lineage. We first ask what cell type is directly produced by Type I and Type II neuroblasts? We found that type I neuroblasts in the DAL region always segregate Pros protein into the newborn GMC [100%, n=9; Figure 3(A)] resulting in easily detectable levels of Pros in neuroblast progeny [see Fig. 3(B)]. Thus, Type I neuroblasts in the DAL generate nuclear Pros+ GMCs, as previously reported (Spana and Doe, 1995; Bello et al., 2006; Betschinger et al., 2006; Lee et al., 2006c). In contrast, Type II neuroblasts of the DPM region often fail to segregate Pros protein [50%; n=14; Fig. 3(C)], despite proper localization of other apical/basal proteins [100%; n=14; Fig. 3(C)], which would account for reduced Pros levels in newborn progeny [Fig. 1(C), arrows]. The variation in Pros localization among DPM neuroblasts could be due to the presence of some Type I neuroblasts in the region, or actual variation among Type II neuroblasts. We conclude that Type II neuroblasts divide asymmetrically, but can fail to segregate Pros protein into their newborn progeny (see Discussion).

We next investigated the relationship between the Type II small cells that have high Dpn, low Pros (Dpn+ $Pros^{cyto}$) and those that contain high Pros, but no Dpn (Dpn– $Pros^{nucl}$). We found that mitotic Dpn+ small cells always form Mira/Pros cortical crescents [100%, n=50; Fig. 3(D)], with Pins protein localized to the opposite cortical domain [100%, n=18; Fig. 3(D)], and the spindle aligned along this cortical polarity axis (data not shown). This type of division is unique to Type II lineages, as all Type I GMCs

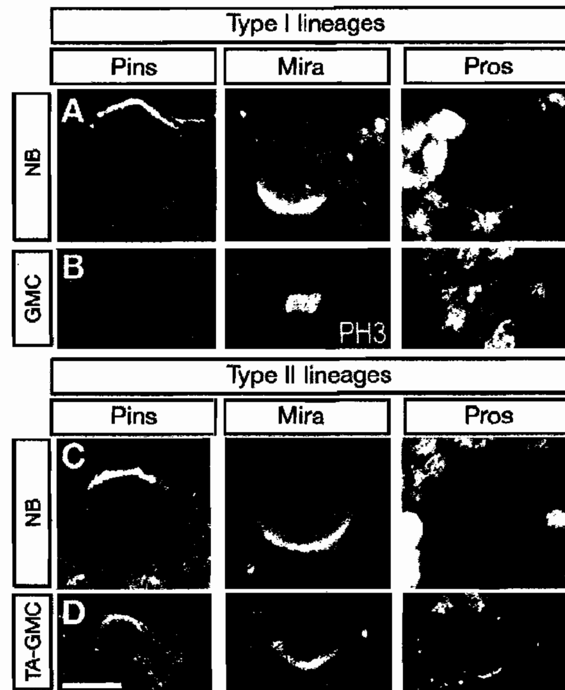

Figure 3 Asymmetric cell division within type I and type II neuroblast lineages. Mitotic larval neuroblasts and GMCs stained for the apical protein Partner of Inscuteable (Pins) and the basal proteins Miranda (Mira) and Prospero (Pros), in some cases the mitotic marker phosphohistone H3 (PH3) is shown. Pros/Mira or Pros/PH3 panels always show the same neuroblast. A: Type I mitotic neuroblast in the DAL region shows apical Pins and basal Mira/Pros. Pros/Mira panels show the same NB. B: Type I mitotic GMC in the DAL region shows diffuse cytoplasmic Pros (bright punctate staining in the Pros panel is DNA-associated Pros protein). C: Type II mitotic neuroblast in the DPM region identified by lack of asymmetric Pros localization, despite completely normal localization of Pins and Mira. Pros/Mira panels show the same NB. D: Type II mitotic TA-GMCs in the DPM identified by their small size and asymmetric localization of Pros and Mira to the cortex opposite the Pins. Scale bar= 6.24 um.

always showed diffuse cytoplasmic Pros during mitosis [100%, n=6 in DAL; Fig. 3(B)]. We conclude that Type II Dpn+ small cells undergo molecularly asymmetric cell divisions to generate a Pros+ sibling and a Pros- sibling. We propose that the sibling with little or no Pros remains a Dpn+ TA-GMC, whereas the Pros+ sibling generates one or two post-mitotic neurons, similar to Pros+ GMCs in Type I lineages (see Discussion).

Type II Neuroblast Progeny are Proliferative but can Generate Differentiated Neurons

To characterize the cell cycle kinetics of Type I GMCs and Type II TA-GMCs, we performed BrdU labeling experiments. We exposed larvae to a 4.5 h BrdU pulse and then immediately fixed and assayed for BrdU incorporation. As expected, both Type I and Type II neuroblasts always incorporated BrdU [Fig. 4(A,C); arrow]. Type I neuroblasts showed only a few closely-associated GMCs labeled [Fig. 4(A); bracket], whereas Type II neuroblasts had a much larger number of labeled progeny [Fig. 4(C); bracket)]. It is unlikely that the Type II neuroblasts generate all of these progeny during the 4.5 h labeling window, because the shortest neuroblast cell cycle time we have observed in any brain region is ~50 min (C. Cabernard and C.Q.D., unpublished results), and thus we conclude that Type II neuroblast progeny undergo more rounds of cell division that Type I GMCs.

To determine if the proliferative Type II neuroblast progeny are competent to differentiate into neurons, we performed a BrdU pulse/chase experiment. Larvae were fed BrdU for 4.5 h as described above, but then allowed to develop for 18 h without BrdU. Type I neuroblasts lacked BrdU incorporation, as expected due to label dilution

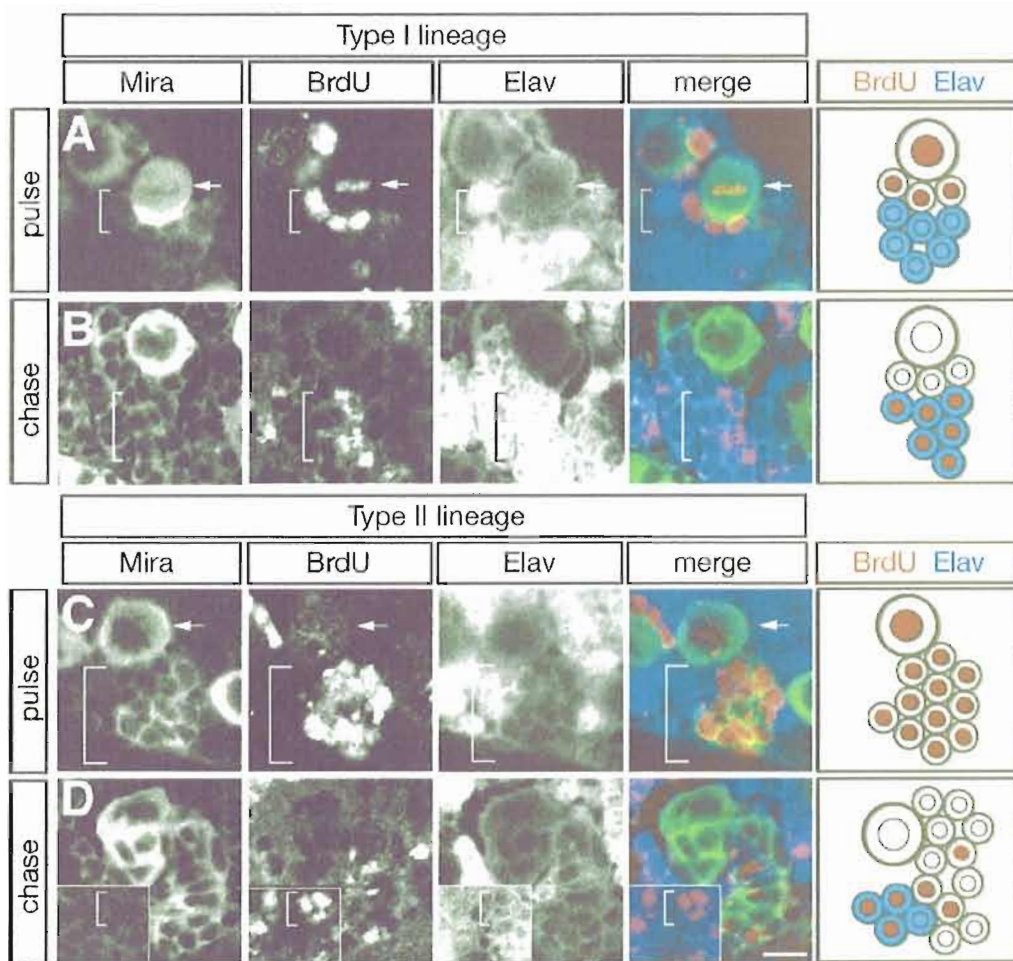

Figure 4 BrdU pulse/chase analysis of type I and type II neuroblast lineages. Larvae were pulsed with BrdU for 4.5 h and then either fixed immediately (''pulse''; A, C) or grown without BrdU for 18 h before fixing (''chase''; B, D). Larvae were stained for Miranda (Mira, green), BrdU (red), and the neuronal marker Elav (blue); neuroblasts (NBs), arrows; neuroblast progeny, brackets; schematics are shown to the right. Type I lineage data were collected in the DAL brain region; type II lineage data were collected in the DPM brain region. (A-B) Type I neuroblasts always incorporate BrdU during the pulse and dilute it out during the chase, whereas only a few type I GMCs contacting the neuroblast incorporate BrdU during the pulse (A, brackets); following the chase, BrdU is maintained in Elav+ post-mitotic neurons (B, brackets). C,D: Type II neuroblasts always incorporate BrdU during the pulse and dilute it out during the chase; many type II progeny incorporate BrdU during the pulse (C, brackets); following the chase, BrdU is maintained in Elav+ post-mitotic neurons (D, brackets; shown in an inset, because the neurons are at the bottom of this confocal image stack). Scale bar= 6.24 lm.

during the chase interval, but BrdU was maintained in the Elav1 post-mitotic neurons born during the pulse window [Fig. 4(B); bracket]. Type II neuroblasts and most of their progeny also diluted out BrdU, confirming their status as proliferative cells (see above), and some Elav1 post-mitotic neurons were born during the pulse interval and maintained BrdU labeling [Fig. 4(D); bracket]. We conclude that Type II neuroblast progeny are proliferative but can still give rise to differentiated neurons.

DISCUSSION

We have identified a novel ''Type II'' neuroblast line-age within the Drosophila larval brain (see Fig. 5). Although we have not documented this lineage by time-lapse imaging, we have the following evidence for each step in the lineage (steps marked by numbers in Fig. 5):

1. Type II neuroblast → Dpn- $Pros^{cyto}$ TA-GMC. We place the Dpn- $Pros^{cyto}$ TA-GMC as the newborn progeny because this is the only cell type always observed in direct contact with the neuroblast, and because the neuroblast can divide without segregating Pros protein into the newborn GMC (consistent with the low levels of Pros in the Dpn- $Pros^{cyto}$ TA-GMC).
2. Dpn- $Pros^{cyto}$ TA-GMC → Dpn+ $Pros^{cyto}$ TA-GMC. We propose that Dpn is rapidly up-regulated in the newborn TA-GMC because (a) Dpn+ small cells are often located close to the neuroblast; (b) pros mutant type I GMCs will up-regulate Dpn levels (Bello et al., 2006; Betschinger et al., 2006; Lee et al.,

2006c); and (c) in other regions of the CNS Dpn protein is detected in mitotic progenitors and not post-mitotic neurons (Bier et al., 1992).

3. All Dpn+ $Pros^{cyto}$ small cells divide asymmetrically to generate one Pros+ cell and one Pros- cell. We propose that the Pros- cell remains a TA-GMC.
4. Dpn- $Pros^{nucl}$ GMC divides to form two post-mitotic neurons. This part of the lineage is based on analogy with Type I GMCs, which have nuclear Pros and divide symmetrically to generate two neurons (Spana and Doe, 1995). Consistent with this model, we can observe small Dpn- Pros+ cells dividing symmetrically with cytoplasmic Pros closely associated with the pool of Dpn+ TA-GMCs in the DPM. Nevertheless, it remains possible that some or all Dpn- $Pros^{nucl}$ cells directly differentiate into neurons.

The most striking feature of the Type II lineages is that they contain TA-GMCs that have features of both neuroblasts and GMCs. TA-GMCs resemble neuroblasts in containing nuclear Dpn, low levels of cytoplasmic Pros, their ability to asymmetrically localize Pros during mitosis, and their ability to divide multiple times; yet they resemble GMCs in their small size, physically symmetric cell division, and relatively limited mitotic potential.

There are currently no molecular markers that can be used to unambiguously identify Type II neuroblasts. The inability to form Pros crescents may be shared by all Type II neuroblasts, but even so, it would only be a useful marker for mitotic neuroblasts.

In the DPM brain region (enriched for Type II lineages) we find about 50% of the mitotic neuroblasts have little or no Pros crescent, and based on the distinctive lack of Pros in some Type II neuroblast progeny, we conclude that these are Type II neuroblasts. (The 50% of the DPM neuroblasts that form Pros crescents may be Type I neuroblasts within the region, a special subset of Type II neuroblasts, or there may be stochastic variability in Pros crescent-forming ability among Type II neuroblasts.) In any case, our findings may explain why some labs report seeing Pros crescents (Bello et al., 2006; Betschinger et al., 2006; Choksi et al., 2006; Lee et al., 2006c) whereas others report that neuroblasts do not form Pros crescents (Ceron et al., 2001); both are correct because there are two types of larval neuroblast lineages.

It is unknown whether neuroblasts can switch back and forth between Type I and Type II modes of cell lineage. If the level of Pros in the neuroblast is the key factor distinguishing these modes of division, then experimentally raising Pros levels in Type II lineages may switch them to Type I lineages; conversely, reducing Pros levels in Type I lineages may switch them to Type II lineages. As more brain neuroblasts become uniquely identifiable it will be interesting to address this question. It will also be interesting to search for Type II neuroblast lineages in other insects or crustaceans where Type I neuroblast lineages have been documented (Goodman and Spitzer, 1979; Goodman and Doe, 1993; Ungerer and Scholtz, 2007). What terminates the TA-GMC lineage? The TAGMC may fall below a size threshold for continued proliferation. Alternatively, TA-GMCs may lose contact with a niche-derived signal that maintains their proliferation; Hedgehog, Fibroblast growth factor (Park et al., 2003), and Activin

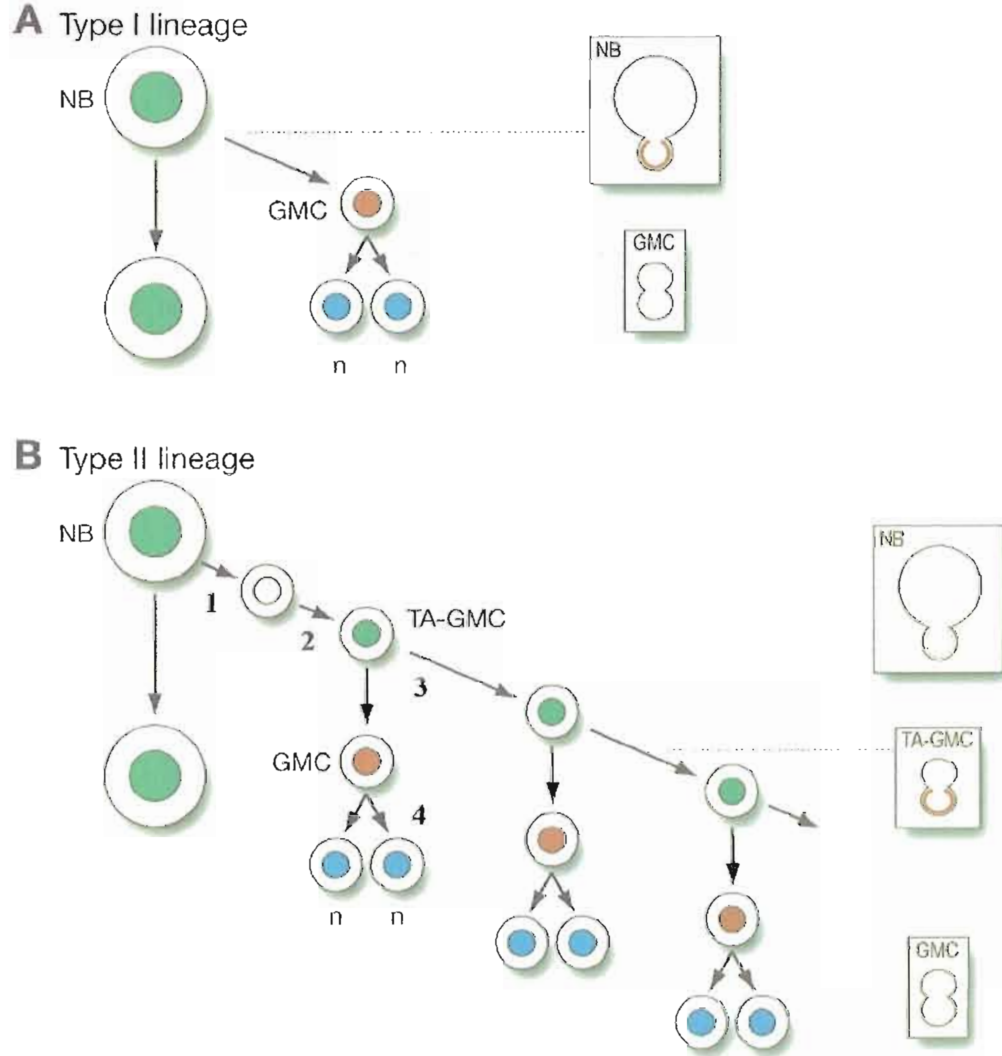

Figure 5 Summary of type I and type II larval neuroblast lineages. A: Proposed type I neuroblast lineage. Nuclear Dpn (green), nuclear Pros (red), cytoplasmic or undetectable Pros (light red), cortical Prospero (red crescent), neuronal marker Elav (blue). Mitotic profiles are shown in boxes at right. See text for details. B: Proposed type II neuroblast lineage. Nuclear Dpn (green), nuclear Pros (red), cytoplasmic or undetectable Pros (light red), cortical Prospero (red crescent), weak or undetectable cortical Pros (dashed red crescent), neuronal marker Elav (blue). Mitotic profiles are shown in boxes at right. See Discussion for details of each numbered step in the lineage.

(Zhu et al., in press) are all required for larval brain neuroblast proliferation, but none have been tested for a role in TA-GMC proliferation. Lastly, there may be lineage-specific factors segregated into the TA-GMCs that limit their mitotic potential. TA-GMCs may die at the end of their lineage, as do some neuroblasts (Bello et al., 2003), or they may differentiate.

It is unknown whether neuroblasts can switch back and forth between Type I and Type II modes of cell lineage. If the level of Pros in the neuroblast is the key factor distinguishing these modes of division, then experimentally raising Pros levels in Type II lineages may switch them to Type I lineages; conversely, reducing Pros levels in Type I lineages may switch them to Type II lineages. As more brain neuroblasts become uniquely identifiable it will be interesting to address this question. It will also be interesting to search for Type II neuroblast lineages in other insects or crustaceans where Type I neuroblast lineages have been documented (Goodman and Spitzer, 1979; Goodman and Doe, 1993; Ungerer and Scholtz, 2007). What terminates the TA-GMC lineage? The TA-GMC may fall below a size threshold for continued proliferation. Alternatively, TA-GMCs may lose contact with a niche-derived signal that maintains their proliferation; Hedgehog, Fibroblast growth factor (Park et al., 2003), and Activin (Zhu et al., in press) are all required for larval brain neuroblast proliferation, but none have been tested for a role in TA-GMC proliferation. Lastly, there may be lineage-specific factors segregated into the TA-GMCs that limit their mitotic potential. TA-GMCs may die at the end of their lineage, as do some neuroblasts (Bello et al., 2003), or they may differentiate.

It has been shown that loss of Pros and Brat together can generate a more severe neuroblast tumor phenotype than either alone (Betschinger et al., 2006). This suggests that the Type II lineages may be especially sensitive to further loss of differentiation promoting factors due to their low levels of endogenous Pros. Indeed, we have observed a dramatic neuroblast tumor phenotype in type II lineages in lethal giant discs mutants (J.Q.B. and C.Q.D., in preparation). This raises the question of how Type II lineages benefit the fly. They have the ability to generate more neurons in a faster period of time, due to the presence of TA-GMCs, and may be an evolutionary adaptation to the rapid life cycle of Drosophila. Slower developing insects may not require such rapid modes of neurogenesis.

Bridge to Chapter IV

Chapter III discussed a novel central brain neuroblast lineage that contains transit amplifying ganglion mother cells. Chapter III further characterizes how this novel neural stem cell lineage arises and how the cells within the lineage behave. This newly defined population of neural stem cells will be important for examining the mechanisms of stem cell self renewal and will aid in our understanding of mammalian transit amplifying progenitor proliferation. Chapter IV will present preliminary data on the molecular mechanisms regulating stem cell self renewal within the type II lineage.

CHAPTER IV

DROSOPHILA LETHAL GIANT DISCS AND *LETHAL GIANT LARVAE* PROMOTE NEUROBLAST DIFFERENTIATION

Jason Q. Boone, Chris Q. Doe. In Preparation. Copyright 2008.

INTRODUCTION

In mammals transit amplifying cells are progeny of slowly dividing tissue specific stem cells (Waters et al., 2007). Here transit amplifying cells divide more rapidly that stem cells and are thus able to generate larger numbers of mature cells within tissues. Importantly tumor formation has been hypothesized to originate within many different stem and progenitor populations (Reya et al., 2001). Since transit amplifying progenitors proliferate more rapidly, loss of proliferation control mechanisms in this population could result in tumor formation. However, work in mammals has been hindered by the inability to individually identify transit amplifying progenitors apart from their parent stem cells. Therefore progress in our understanding the mechanisms that regulate normal and tumor growth within lineages that contain stem and transit amplifying progenitors has been slow.

Neuroblasts are the neural stem cells of the *Drosophila* central nervous system. Neuroblasts have become a useful model system for understanding the mechanisms regulating tissue specific stem cell self renewal (Lee et al., 2006). Interestingly specific neuroblast lineages within the larval central nervous system have been shown to contain transit amplifying progenitors. Canonical neuroblast lineages produce one neuroblast that continues to self renew and one ganglion mother cell that divides once to produce two neurons (Doe, 2008). Non-canonical lineages also produce a ganglion mother cell that re-expresses neuroblast markers and divides in a self renewing fashion to produce multiple neurons (Bello et al., 2008; Boone et al., in press; Boweman et al., 2008).

TA-GMCs provide a useful model system to tease apart the mechanisms regulating transit amplifying cell proliferation from those regulating neuroblast proliferation. TA-GMCs arise from insufficient Prospero segregation into the newborn gmc and can be identified as small Deadpan (Dpn), Asense (Ase) positive cells that reside in specific larval brain locations (Boone et al., in press; Boweman et al., 2008). TA-GMCs are proliferative and can generate up to 10 neurons, however, the mechanisms controlling their proliferation and self renewal are currently unknown.

It has recently been suggested that several mutants exhibiting larval brain tumors arise from over proliferation and or defective self renewal within TA-GMC populations (Boweman et al., 2008). They suggest that loss of *brain tumor* (*brat*) and *lethal giant larvae* (*lgl*) affect non-canonical lineages more so than canonical lineages. Furthermore, they show that over-expression of Notch in all brain neuroblasts yields over production of

TA-GMCs and that loss of function Notch signaling precludes non-canonical lineages from transitioning to the TA-GMC fate.

Since Notch signaling is important for neuroblast self renewal and TA-GMC self renewal we utilized an allele of lethal giant discs (*lgd*), as *lgd* has been shown to regulate Notch endocytosis and signaling (Childress et al., 2006; Gallagher et al., 2006; Jaekel et al., 2006). I show here that the allele *lgdd7* contains a background mutation *lgl*, and that *lgdd7, lgl* double mutants have TA-GMC tumors. Further I show that a large tumor of TA-GMCs start from early larval stages and originates in the dorsoposterior medial (DPM) region, which is also the location of non-canonical TA-GMC lineages. Lastly I show that neuroblast tumors in *lgdd7, lgl* can proliferate but do not make maturing progeny. Based on these data I conclude that both *lgd* and *lgl* are necessary for non-canonical lineage neuroblast self-renewal as well as ordered progression through the lineage.

MATERIALS AND METHODS

Fly stocks

Oregon R flies were used as wild type. *lgl334* and *lgdd7d, lgl* (kind gift from Thomas Klein)

Immunostaining and confocal analysis

Larval brains were dissected in Schneider's medium (Sigma, St. Louis, MO); fixed in 100 mM Pipes (pH 6.9), 1 mM EGTA, 0.3% Triton X-100, and 1 mM MgSO4 containing 4% formaldehyde for 25 min; washed 30 min in phosphate buffered saline (PBS); washed 30

min in PBS containing 0.3% Triton X-100 (PBS-BT); and incubated with primary antibodies in PBS-BT overnight at 4°C. Primary antibodies were rat Dpn monoclonal (1:1), mouse Pros monoclonal (purified MR1A, 1:1000), mouse BrdU (1:50; Sigma, St. Louis, MO) and rat Elav (1:10; DSHB). Secondary antibodies were from Molecular Probes (Eugene, OR). Antibodies without named sources were made in the laboratory; details are available on request. Images were captured with a Biorad Radiance or Leica SP2 confocal microscope and processed in Photoshop 7 (Adobe, San Jose, CA).

BrdU pulse/chase experiments

Bromodeoxyuridine (BrdU) was purchased from Roche (Basel, Switzerland), dissolved in 1:1 DMSO:acetone, and mixed with food media at a final concentration of 1 mg/mL. Larvae were fed on BrdU-containing food for 4.5 h and immediately fixed for pulse experiments, or allowed to develop on food lacking BrdU for 18h before fixation for chase experiments. Larval brains were dissected, fixed, and antibody stained as described above with the addition of a 2N HCl treatment for 30 min prior to BrdU staining.

RESULTS

lethal giant discs **Allele** ***lgdd7*** **contains a background mutation in lethal giant larvae**

lethal giant discs (*lgd*) has recently been shown to negatively regulate Notch signaling through endocytosis of the Notch receptor (Childress et al., 2006; Gallagher et

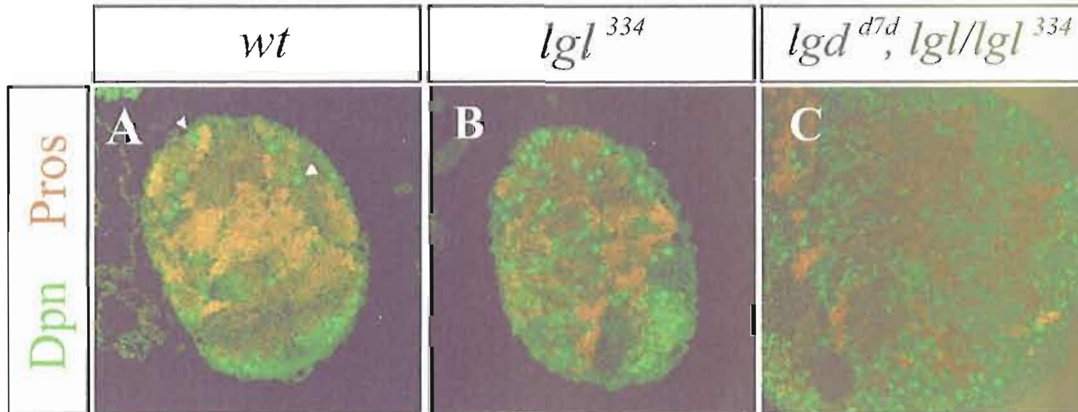

Figure 1 *lgdd7d* contains a background mutation in *lgl*. Wild type (wt) *lgl* L3 larvae and *lgdd7d, lgl/lgl* 160 hours after larval hatching larvae brain confocal sections: Anterior to top posterior to bottom and lateral to the right. (A) Individual canonical neuroblasts can be seen as large Deadpan (Dpn, green) positive and Prospero negative cells; Non-canonical neuroblast lineages cannot be seen in this section. (B) *lgl334* mutants have many more Dpn positive neuroblasts with Prospero positive gmcs interspersed; mutants die at late larval, early pupal stages. (C) *lgl* fails to complement *lgdd7d* by lethality and expanded number of Dpn positive neuroblasts similar to *lgl334* single mutants. The number of neuroblasts cannot be compared between B and C as stages are not similar.

al., 2006; Jaekel et al., 2006). To further understand how Notch signaling controls canonical and non-canonical neuroblast lineage self renewal we utilized the *lgdd7* allele previous published to be a null mutation of *lgd* and also gives a strong loss-of-function Notch phenotype in the peripheral nervous system (Gallagher et al., 2006; Jaekel et al., 2006).

We examined neuroblast number in late larval *lgdd7* mutants and found there to be too many neuroblasts per brain lobe at all stages examined (see next section). However, in collaboration with Dr Gary Struhl, we found that an independent allele of *lgd* also termed "*lgdd7*" did not generate as severe an increase in neuroblast number as my *lgdd7* allele. At this point Dr. Struhl and I termed my *lgdd7* allele "*lgdd7d*" and his *lgdd7* allele "lgdd7." These data suggested that one of the *lgdd7* alleles contained a background mutation. Concordantly data from microarray experiments (M. Miller and C.Q. Doe unpublished) suggested that *lgdd7d* contained no transcript for the gene *lethal giant larvae (lgl).* Since *lgl* is located at the proximal end of chromosome 2L, an entire portion of the end of chromosome 2L may have been lost in the *lgdd7d* allele. Indeed the spontaneous mutation *lgl334* is considered to have arisen by complete loss of the distal end of chromosome 2L, possibly including surrounding genes (Doe CQ, unpublished observation). Therefore we examined the surrounding genes transcript levels in the *lgdd7d* microaray experiments. Only one unknown gene, CG31973, had central nervous system expression and showed transcript levels on the *lgdd7d* microarray.

To test genetically whether *lgdd7d* contained a background mutation in *lgl* I performed a complementation test with *lgdd7d* and *lgl334* and compared these results to *lgl334* homozygous mutants. *lgl334* homozygous mutants are lethal at larval stages and do not produce adults (Bryant et al., 1971). Furthermore, *lgl4* homozygous mutants produce too many brain neuroblasts at larval stage L3 (Bryant et al., 1971). Indeed I show that *lgl334* homozygous mutants do contain too many brain neuroblasts at L3 compared to wt (Figure 1A, B). When I performed the complementation test, I found that *lgdd7d/SM6, TM6B* mutants crossed with *lgl334/Cyo, GFP* yield only balanced adults indicating failure of *lgl334* to complement *lgdd7d.* When late stage larval brains were examined we found an increase in the number of larval brain neuroblasts (Figure 1B, C) reminiscent of *lgl334* homozygous mutants. The number of neuroblasts cannot be compared between *lgl334* homozygous mutants and *lgdd7d/lgl334* mutants as the *lgdd7d/lgl334* mutants were examined at a far later developmental stage. Accurate analysis of neuroblast numbers must be assayed at similar developmental stages.

These data suggest that the *lgdd7d* allele contains a background mutation in *lgl* and surrounding genes. At this time, it is unknown whether the surrounding genes have any influence on the *lgl* phenotype in the *lgdd7d* background. From now on we will call the *lgdd7d* allele, *lgdd7, lgl.*

lgdd7, lgl Double Mutant Brains Contain TA-GMC Tumors

While assaying complementation and neuroblast numbers in *lgdd7, lgl* double mutants we found two distinct phenotypes that were more pronounced at late stages of

larval life compared to earlier stages. First, we found large Dpn-positive-only clusters of neuroblasts (Figure 2B, dotted lines). The number of cells within the large neuroblast only cluster begins increasing dramatically from at 48 hours after larval hatching (ALH). The cluster can clearly be identified at early stages in the dorsal medial region of the brain lobe (Figure 2D). Over time this cluster of neuroblasts grows consuming more than two-thirds of the brain lobe by 96 hours ALH (Figure 2B). These large neuroblast clusters are not optic lobe neuroblasts or transformed optic lobe epithelium; they are located on the medial half of the brain at early stages, whereas optic lobe neuroblasts are more lateral, residing near the optic lobe epithelium. Interestingly many of the Dpn positive cells within the large cluster are much smaller than canonical neuroblasts and are much more similar to the size of TA-GMCs (roughly 5-7 um) (J.Q.B unpublished observation). Since the large Dpn positive clusters arise in the dorsal posterior medial region of the brain and many of the cells within the cluster are similar in size to TA-GMCs I consider this large cluster to be a TA-GMC tumor incapable of reactivating and asymmetrically segregating prospero.

Second we found small clusters or small single Dpn positive neuroblasts scattered throughout the brain around the larger Dpn+ clusters (Figure 2B, D arrowheads). These neuroblasts were often much smaller than wild type neuroblasts but not as small as TA-GMCs and were more randomly interspersed throughout the brain (Figure 2B, D arrowheads).

To distinguish between these two phenotypes we examined the extent of differentiation in each category described above by looking for Pros positive maturing

gmcs and neurons in proximity to neuroblasts. In wild-type brains neuroblasts produce gmcs that will divide once to make neurons or glia. GMCs inherit Pros protein and RNA from the parent neuroblast and express low to high levels of Pros during their transition to maturing neurons (Figure 2A, C). Maturing neurons down-regulate Pros and become Elav positive and are often located in close proximity to gmcs with projections traversing towards the central neuropile. Non-canonical neuroblast lineages begin with a neuroblast that does not asymmetrically segregate sufficient Pros and generates a Pros/Dpn double negative sibling. This sibling then matures to produce a small Dpn positive TA-GMCs that will asymmetrically segregate sufficient levels of Pros to produce a canonical gmc (Bello et al., 2008; Boweman et al., 2008; Boone et al., in press). In *lgdd7, lgl* double mutant brains we observe two distinct phenotypes: 1) large clusters of neuroblasts that do not contain Pros positive cells (Figure 2 B, D inside dotted lines) and 2) smaller clusters or single neuroblasts with Pros positive gmcs surrounding (Fig 2B, D outside dotted lines).

To determine the number of brain neuroblasts in double mutants compared to wt, I counted individual Dpn positive cells in each brain lobe. The number of larval neuroblasts during each larval stage in wild-type and double mutant are shown in chart 1, (Figure 2E). At 24 hours after larval hatching (ALH) there are 26 neuroblasts increasing to 98 at 96 hours ALH. When we examined *lgdd7d, lgl* double mutant brains the overall number of neuroblasts in mutants was greater than wild type at every stage examined. At 24 hours ALH there are 150 neuroblasts, whereas at 96 hours ALH there are 350 (Figure

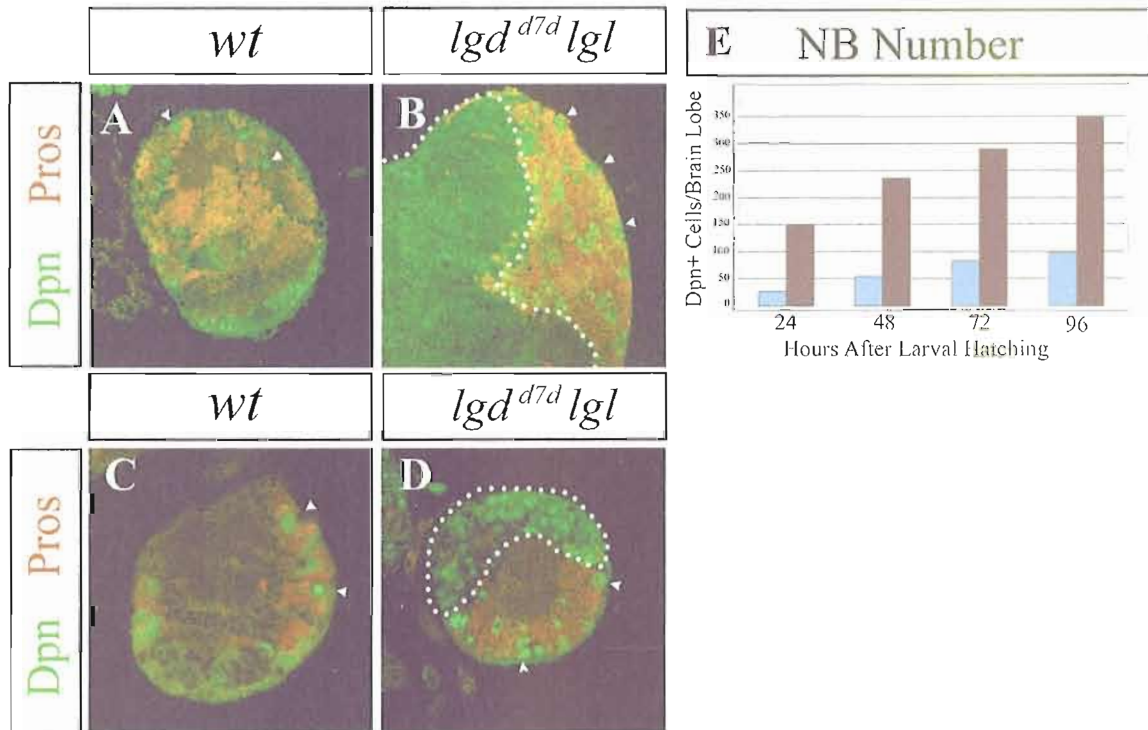

Figure 2 *lgdd7d, lgl* double mutants contain ta-GMC tumors. Two distinct phenotypes and neuroblast numbers in wild type (*wt*) and *lgdd7d, lgl*. (A) *wt* confocal brain section of late L3 larvae; individual canonical neuroblasts can be seen as large Deadpan (Dpn, green; arrowheads) positive, Prospero (Pros, red) negative cells. At late stage L3, *wt* larval brains contain approximately 98 neuroblasts (E). (B) *lgdd7d, lgl* late stage L3 double mutants have two distinct phenotypes: 1) large Dpn only clusters of neuroblasts (dotted outline) without Pros positive gmcs and 2) individual or small clusters of Dpn positive cells (arrowheads) surrounded by small Pros positive gmcs. *lgdd7d, lgl* double mutants contain approximately 350 Dpn positive neuroblasts (E). (C) *wt* confocal brain section of early L2 larvae; individual neuroblasts can be seen as large Dpn positive cells (arrowheads). At early L2, *wt* brains contain approximately 60 neuroblasts (E). (D) *lgdd7d, lgl* late stage L3 double mutants have two distinct phenotypes similar to late stages (B). Large Dpn positive clusters can be identified and localized to the dorsal posterior medial brain lobe region (dotted outlines)

2E). These data suggest that the number of neuroblasts starts off too high in double mutants and increases further over larval life.Taken together these results suggest that *lgdd7d, lgl* double mutants have too many neuroblasts at all stages. There are two distinct phenotypes: 1) large neuroblast- only clusters and 2) small cluster of neuroblast surrounded by GMCs. That the large neuroblast-only cluster grows from the dorsal posterior medial brain region and type II lineages originate in the dorsal posterior medial region and many neuroblast in the large cluster are small, I conclude that type II lineages are the source of the large neuroblast cluster. Furthermore, I conclude that type II lineages are more susceptible to loss of *lgdd7d* and *lgl* than type I lineages.

lgdd7, lgl Double Mutant TA-GMC Tumors Proliferate but Do Not Produce Neurons

To further characterize why *lgdd7d, lgl* double mutant brains have excessive neuroblasts we performed BrdU pulse/chase experiments. Briefly, BrdU is allowed to incorporate into proliferating cells for a period of 4 hours. Larval brains were either dissected directly or aged for 18 hours. After a 4 hour incubation, BrdU is incorporated into proliferating neuroblasts and the first gmcs (pulse) (Figure 3A). However, by 18 hours after the initial incorporation most BrdU has passed into differentiating Elav positive maturing neurons (Figure 3C). In *lgdd7d, lgl* brains I found that during the pulse period individual and clusters of neuroblasts all incorporate BrdU (Figure 3B dotted lines and arrowheads). However in contrast to wild type, during the chase period we find that large clusters of neuroblasts always retain low levels of BrdU and never pass it to nearby

gmcs or neurons (Figure 3D dotted lines). Interestingly however in *lgdd7d, lgl* double mutants, small individual neuroblasts pass BrdU into maturing Elav positive neurons (Figure 3D arrows) similar to wild type (Figure 3B arrows). Taken together these data indicate that *lgdd7d, lgl* double mutants have too many neuroblasts that self renew excessively forming neuroblast tumors most likely in dorsal posterior medial brain regions where TA-GMC lineages exist.

DISCUSSION

I have shown that the *lgdd7* allele harbors a background mutation in *lgl.* Although I have not documented a direct comparison of neuroblast numbers between *lgl* and *lgd* single mutants compared to double mutants I can make the following conclusions:

1). *lgl* single mutants die at or shortly after pupal stages with enlarged brains full of neuroblasts. *lgdd7d/lgl334* trans-heterozygotes also die at or shortly after pupal stages with brains full of neuroblasts. Therefore *lgl* fails to complement *lgdd7d* indicating that the *lgdd7d* allele has a background mutation in *lgl.*

2) Many of the ectopic neuroblasts in *lgl334* and *lgdd7d, lgl* are much smaller than individual brain neuroblasts. Further, in *lgdd7d, lgl* double mutants, the neuroblast-only cluster arises from the dorsal posterior medial brain region where non-canonical lineages and TA-GMCs reside. Therefore with these preliminary data I conclude that the large neuroblast cluster found in double mutants originates in non-canonical type II lineages yielding many large and small Dpn positive neuroblasts and TA-GMCs.

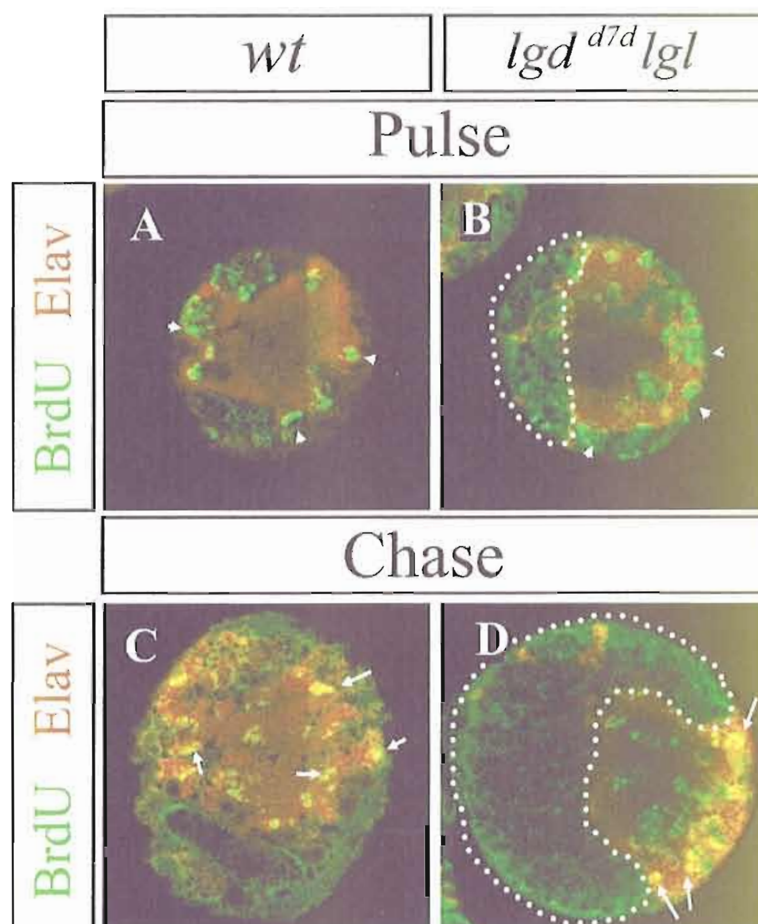

Figure 3 BrdU Pulse/chase analysis in Wild type (wt) and *lgdd7d, lgl*; 48-72 hours after larval hatching confocal brain sections: Anterior up, posterior down and lateral to the right. Larvae were pulsed with BrdU for 4.5 h and then either fixed immediately (''pulse''; A, B) or grown without BrdU for 18 h before fixing (''chase''; C, D). Larvae were stained for the neuronal marker Elav (Elav, green), BrdU (red); neuroblasts, arrowheads and dotted outlines; neuroblast progeny, arrows. (A, C) *wt* neuroblasts always incorporate BrdU during the pulse and dilute it out during the chase, whereas only a few gmcs contacting the neuroblast incorporate BrdU during the pulse (A, arrowheads); following the chase, BrdU is maintained in Elav post-mitotic neurons (C, arrows). (B) *lgdd7d, lgl* double mutants show two phenotypes in BrdU pulse assay 1) large Dpn positive clusters of neuroblasts located in the dorsal posterior medial region all incorporate BrdU (B, dotted outline) and 2) individual brain neuroblasts and few gmc progeny in other regions incorporate BrdU (B, arrowheads). (D) *lgdd7d, lgl* double mutants show two phenotypes in BrdU chase assay 1) large clusters of Dpn positive neuroblasts in the dorsal posterior medial brain region all pass BrdU into more Dpn positive neuroblasts with no Elav positive maturing neurons located within the cluster (D, dotted outlines) and 2) brain neuroblasts in other regions pass BrdU into maturing Elav positive neurons (D, arrows)

Why might non-canonical type II lineages of the dorsal posterior medial region produce a much larger neuroblast-only cluster than canonical lineages in double mutants? Type II lineages have been shown to have insufficient asymmetric segregation of Prospero, a cell fate determinant necessary for differentiation of gmcs. Further it has been shown that loss-of-function *prospero* MARCM clones contain ectopic Dpn positive neuroblasts, many of which are smaller than the parent neuroblast (Bello et al., 2006). These data suggest that upon reduction of Pros, Dpn can reactivate in the newborn gmc, producing a gmc that divides multiple times. With limited Pros segregation into the newborn type II gmc, part of the mechanism directing differentiation is absent. I believe the absence of Pros segregation leaves type II lineages more vulnerable to loss of tumor suppressors such as *lgl* and *lgd*. Taken together this suggests that Lgd and Lgl function in type II lineages to promote differentiation.

How might Lgd and Lgl act to promote differentiation within type II lineages? *lgd* has recently been shown to inhibit Notch signaling in the peripheral nervous system and in the wing disc (Childress et al., 2006; Gallagher et al., 2006; Jaekel et al., 2006). Since Notch is necessary and sufficient for neuroblast self renewal, I hypothesize the role of *lgd* in regulating neuroblast self renewal is through Notch signaling. I will address this issue with further experiments.

Lgl is a component of the cortical polarity pathway in both epithelium and neuroblasts. Lgl has been shown to regulate the localization of the Notch inhibitor, Numb in sensory organ precursor cells within the peripheral nervous system (Langevin et al., 2005). It is likely that by acting as a component of the cortical polarity pathway, Lgl

regulates Numb localization in neuroblasts as well. I will address this issue with further experiments.

How does Notch regulate self renewal within the type II lineages? It has been shown that loss-of-function *N* leads to delayed differentiation within type II lineages (Boweman et al., 2008). It has also been shown that over activation of Notch causes a TA-GMC tumor like phenotype. These data suggest that *N* is necessary and sufficient for type II lineage neuroblast and TA-GMC self renewal. Exactly how Notch achieves this is unclear.

Bridge to Chapter V

Chapter IV introduced preliminary data on the molecular mechanisms regulating differentiation within type II transit amplifying lineages. Chapter IV further shows that *lgd* and *lgl* inhibit TA-GMC differentiation to a greater extent than type I lineages.
The fact that *lgd* and *lgl* can cause tumorous overgrowth in one lineage and not another is of great importance. Chapter IV will summarize the findings of Chapters II, III and IV as well as give further details about the impact of these results.

CHAPTER V

CONCLUSIONS

Optic Lobe Development

Proper development of the *Drosophila* optic lobe is essential for correct visual processing. Development of the lamina within the optic lobe has been well studied to date. However, the development of the medulla within the optic lobe has not been examined in any detail. Here we show that, during larval stages, the optic lobe has two proliferation zones; the outer proliferation center (OPC) and inner proliferation center (IPC). Within the OPC, there are two cell types arranged in two distinct medio-lateral zones: 1) columnar shaped epithelial cells with characteristic junctional complexes and 2) rounded neuroblasts expressing specific neuroblast markers that asymmetrically segregate fate determinants. We show further with MARCM analysis and live imaging that optic lobe epithelial cells undergo proliferative symmetric divisions whereas neuroblasts undergo differentiative asymmetric divisions. To understand the lineage relationships between optic lobe epithelial cells and optic lobe neuroblasts we induced "flipout" clones to track the lineage of single or small clusters of cells. We show that optic lobe epithelial cells give rise to optic lobe neuroblasts in spatially distinct zones; the most medial zones of optic lobe epithelium generate neuroblasts. We show further that

neuroblasts generated at the medial edge of the optic lobe epithelium generate the neurons of the medulla. Lastly, whether misorientation of mitotic spindles within *Drosophila* embryonic neuroepithelium was causal or a consequence of neuroblast segregation has remained unclear. We show here that misorientation of the mitotic spindles within the optic lobe epithelium, which is similar to embryonic neuroepithelium, does not induce production of ectopic neuroblasts. These results lead us to conclude that the optic lobe of Drosophila is generated in a step-wise fashion similar to the vertebrate retina and neural tube: 1) a small group of epithelial cells folds to form a small cluster of cells that will invaginate towards the interior, 2) this small progenitor cluster begins to proliferate in a symmetric division profile to increase the pool size of neuronal progenitors, 3) at later stages more restricted neuronal progenitors (neuroblasts) are generated in specific zones, 4) neuroblasts divide in an asymmetric fashion to generate neurons within the medulla cortex. This mode of neurogenesis is reminiscent of the vertebrate neural tube and will provide a useful model system to understand how cells transition from symmetric to asymmetric divisions.

Transit Amplifying Neuronal Progenitors

How a neuronal progenitor can produce the myriad different cell numbers and types found within the central nervous system has long intrigued scientists. It has become clear that asymmetric cell division is one fundamental manner in which cells produce different offspring. However, whether this is the fundamental manner in which cells produce different cell types as well as different cell numbers is unclear. Here we show a

novel neural stem cell lineage in *D. melanogaster* that utilizes transit amplifying progenitors to increase the number of neuronal progeny produced. We used clonal analysis to show that the larval brain of Drosophila contains at least two types of neuroblast lineages: 1) A type I lineage that is canonical to embryonic and MB lineages and 2) a type II, non-canonical lineage that contains multiple transit amplifying ganglion mother cells (TA-GMCs). That type II lineages contain transit amplifying ganglion mother cells, is a defining characteristic of this lineage. We show further that transit amplifying lineages are located within specific brain lobe regions. To understand why these specific lineages contain transit amplifying ganglion mother cells we asked whether the parent neuroblast segregates fate determinants into the ganglion mother cell; inability to properly segregate fate determinants into the gmc has been shown to cause ectopic activation of a neuroblast expression pattern within the GMC. Indeed we show that at least 50% of neuroblasts in the region where type II lineages are located, there are large parent neuroblasts that do not segregate the fate determinant Prospero. Finally we show that TA-GMCs are proliferative and can generate neurons. The presence of TA-GMCs within this lineage is interesting because it allows for a greater number of neurons to be produced in a similar time frame than in type I lineages.

In chapter IV I show that type II lineages are more susceptible to loss of tumor suppressors, such as *lgd* and *lgl*. Because type II lineages do not segregate sufficient levels of Pros into the daughter gmc, type II lineages no longer have a dual mechanism ensuring gmc differentiation. What is the nature of the dual mechanism ensuring proper differentiation of canonical GMCs? Wang et al (Wang et al., 2006) have suggested that

the cortical polarity protein aPKC, can direct such a dual mechanisms by regulating the localization of two basal determinants Pros and Numb. In this model they suggest that Pros directly inhibits self renewal genes and Numb inhibits Notch signaling thus ensuring self renewal is fully inhibited in daughter GMCs.

Is it possible that type II lineages are a remnant of more primitive direct developing insects? Currently neurogenesis patterns described above have not been examined in basal arthropods. However, direct developing insects do not go through consecutive stages of crawling larvae; small versions of the adult organism emerge after the embryonic stage and are ready to interact with the environment. Therefore I hypothesize that direct developing insects are ready to interact with their environment after the first molt suggesting that their central nervous system is capable of taking in, processing and generating appropriate responses. Therefore the CNS of more basal arthropods would rapidly develop within the short window of embryonic life in order for the organism to properly interact with their environment. The model of neurogenesis we have uncovered here may be similar to more primitive insects with rapid neurogenesis patterns.

Lastly, by examining the normal developmental mechanisms of different lineages within the larval central nervous system of *Drosophila* I show that neurons are generated in widely different ways. The fact that neurons are generated in different ways suggests that the molecular mechanisms regulating individual lineages may be different. Indeed I show that *lgd* and *lgl* control self-renewal and differentiation within type II non-canonical lineages more so than in type I lineages. These data suggest that some neural stem cell

lineages utilize different mechanisms to generate neurons and therefore are more susceptible to tumor formation upon loss of tumor suppressors. It will be important to examine whether neural stem cell lineages in mammals have lineage sub-types and whether some of these sub-types are more susceptible to loss of tumor suppressors.

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